Detection of DNA Damage:
The Synthesis of Aldehyde Reactive
Probes for the Quantitation of Abasic Sites

(Under the direction of Dr. Avram Gold)

Abstract

Apurinic / apyrimidinic (AP sites) are the result of damage to DNA. These sites can inhibit replication of the DNA and cause mutations. They are considered to be important intermediates in carcinogenesis and in mutagenesis. It is advantageous to have a simple assay capable of detecting and quantifying these sites in a timely manner. Kubo et al. reported such an assay wherein the aldehyde group in the abasic site reacts with an aldehyde reactive probe (ARP). The number of tagged AP sites is then quantified colorimetrically by an assay similar to an ELISA. The purpose of this research project was to synthesize additional reactive probes that could be used in the detection of abasic sites in DNA. ARP was synthesized using the procedure of Ide et al. Procedures for the synthesis of labeled ARP components, *O*-(carboxymethyl)hydroxylamine and biotin hydrazide were developed. Synthetic routes to new ARPs were also investigated. A novel 2-iminobiotin hydrazide hydrochloride probe and a novel hydrazinoacetic acid derivative probe were synthesized and characterized. In addition, a synthetic route to a probe containing a long alkyl tether group was nearly completed.

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I. Introduction

DNA is the building block of life and represents the complete hereditary background of all living things. Given the importance of DNA, genomic responses to certain stressful environmental and chemical threats are becoming increasingly significant as more and more knowledge is gained in the fields of DNA repair and mutagenesis. Further significant contributions to man's knowledge of mutagenesis and carcinogenesis may result from being able to efficiently and sensitively detect damage to DNA. Apurinic/apyrimidinic (AP) sites are common non-coding DNA lesions that can inhibit DNA replication and result in mutations and loss of genetic integrity (Lindahl and Nyberg 1972; Nakamura et al. 1998). AP sites are formed either spontaneously, by direct action of damaging agents, or as intermediates during the course of base excision repair (BER) of oxidized, deaminated, or alkylated bases (Atamna et al. 2000). Detection of AP sites is important since AP sites are components of DNA damage and repair.

In view of the significance of AP sites, several methods have been established for their detection and quantification. However, many are either very labor intensive or require the use of radioactivity or lack sensitivity for measurement of low numbers of AP sites (Ide et al. 1993; Nakamura et al. 1998). Kubo et al. demonstrated a simple, sensitive method for the detection of AP sites using an aldehyde-reactive probe (ARP).

This research focuses on the synthesis of the ARP using t-butyloxycarbonyl (*t*-BOC) as a protecting group coupled with an activated ester synthesis. This research also involves work on the synthesis of other novel ARP probes that could be used in the

detection of AP sites. Modifications of ARP may allow for further improvements in AP quantification assays that can increase sensitivity, specificity, and stability of reagents. The DNA probes involved in this research can be seen in Figure 1.

A novel ARP was synthesized using 2-iminobiotin hydrazide hydrochloride, a compound similar in structure to biotin hydrazide differing only in that it is present as a salt and has an imino rather than a carbonyl group at C2. In the ARP assay, once AP sites have reacted with ARP, the complexes can be detected by the binding of the biotin to avidin or streptavidin, which then, when reacted with the appropriate substrate, will give a signal proportionate to the number of AP sites. The avidin-biotin complex is characterized by a dissociation constant (K_D) of approximately 10-15; streptavidin is reported to have a similar K_D value (Heney and Orr 1980). As such, the binding of avidin-biotin is very strong and makes it difficult to use in the affinity isolation of biotinylated components. Release of complexes from an IA column require harsh conditions which can denature proteins. However, iminobiotin has a much lower dissociation constant that has the additional advantage of being pH dependant. At high pH, the 2-iminobiotin exists mostly in its free base form with tight binding to avidin characteristic of biotin. At lower pH, the compound exists in the salt form, and it interacts only weakly with avidin (Heney and Orr 1980). This ionization of the guanidine group, as shown in Figure 2, makes the purification of compounds conjugated to iminobiotin possible under mild conditions by affinity chromatography (Athappilly and Hendrickson 1997). AP sites tagged with iminoARP can be excised by enzyme and then easily purified by streptavidin affinity chromatography before being quantified by LC/MS.

In addition, a probe was synthesized with tertiary butyl carbazate, thus changing the reacting moiety of the original ARP from $-ONH_2$ to $-NHNH_2$. Much of the technology available today does not allow for the distinction between intact AP sites and AP sites at the 3'-terminus (Malvy et al. 1999). To measure AP sites at the 3'-terminus using ARP requires 5' cleavage with exonuclease III, but non-specific cleavage also occurs at the 3' linkage making the quantification very difficult. Livingston noticed that cleavage of the DNA backbone was caused by compounds containing $NH-NH_2$ and not those containing $O-NH_2$. The formation of a Schiff base enhances the departure of a proton from C2' of the deoxyribose and makes the phosphodiester bond labile (Figure 3) (Talpaert-Borle' and Luizzi 1983). The reaction of hydroxide ions or primary amines with the aldehyde functional group in an AP site leads to 3' cleavage through beta elimination and then to secondary cleavage in 5' through delta elimination which results in the removal of the AP site along with the deoxyribose ring from the DNA backbone (Malvy et al. 2000). It is proposed that hydrazinoARP will cleave 3', requiring no endonuclease, resulting in a more accurate way of measuring intact sites through the number of breaks. Excised sites can then be easily characterized and quantified by LC/MS.

Synthesis of a probe incorporating a long alkyl chain was attempted by conjugating 16-hydroxyhexadecanoic acid to biotin hydrazide. The primary objective of this modification to ARP was to purify olignucleotides containing abasic sites (Krotz 2001). Based on research with methoxyamine (MX), which can inhibit the cleavage of AP sites (Liu et al.1999), the long chain tether may also have some inhibitory effects

towards AP endonuclease. As such, it may have a place among anti-cancer drugs (Taverna et al. 2001).

II. Literature Review

II. A. AP Site Formation

The DNA in each cell contains all the genetic information needed for growth and reproduction. Genes carry the instructions for making proteins, enzymes, and other biomolecules that are responsible for carrying out cellular processes. This information is coded by a series of four bases -adenine, guanine, cytosine and thymine linked together in a specific sequence or code. The sequence in which these molecules are ordered determines the information contained in the DNA (Campbell 1994).

Given the role of DNA, maintenance of the accuracy of the genetic code is critical for the survival of cells, individuals, and even whole species. However, the structure of DNA is dynamic and can be altered in several ways (Friedberg 1995). Genomes are vulnerable to a wide array of DNA damage which comes in many forms and can be caused by numerous agents. Oxidation, hydrolysis, and alkylation are all major forms of damage. Formation of apurinic/apyrimidinic (AP) sites makes up one of the major groups of DNA lesions. AP sites arise via the spontaneous, chemically-induced, or enzyme catalyzed base excision repair (BER) involving hydrolysis of the N-glycosyl bond, separating the base from the sugar backbone in the DNA (Loeb and Preston 1986).

II.A.1. Spontaneous Damage

Spontaneous, or endogenous DNA damage occurs as a result of natural processes in cells. Such damage may occur as a result of the inherent instability of the DNA molecule or through errors in replication or recombination. In addition to AP sites, base modifications, deoxyribose damage, and strand breaks are all examples of spontaneous

changes. (Croteau and Bohr 1997). Premutagenic lesions can result from spontaneous changes in the chemical structure of the bases that alter their capacity for hydrogen bond formation. These changes include tautomeric shifts, deamination, damage from oxidative stress, and depurination and depyrimidination. (Friedberg 1995).

II.A.1.a. Tautomeric Shifts

Tautomeric shifts are the rearrangement of bonds resulting in structural isomerism. All of the bases have tautomer forms which alter their base-pairing characteristics. The 4-keto form of thymine and the 6-keto form of guanine are favored; however, a 1,3-shift of the amido hydrogen results in the enol forms (Wade 1995). The pairing properties of the enol tautomers change so that T-G and G-T mispairing occurs. A small fraction of T and G can always be found in the less stable enol form. If the enol tautomer is present during the replication process, the base will mispair in the daughter strand (Watson 1976).

II.A.1.b. Deamination

Deamination can occur in adenine, guanine, and cytosine and is a type of single base change damage. Deamination occurs when the exocylic amino group is lost from one of the above bases. It can occur either spontaneously in pH and temperature dependent reactions in DNA or be induced by chemicals, such as nitrous acid and sodium bisulfite. (Friedberg 1995).

II.A.1.c. Oxidative Damage

Reactive oxygen species (ROS) are also an important source of spontaneous damage to DNA. (ROS) are generated in cells during normal metabolic processes and by a number of exogenous agents, including ionizing radiation (Nakamura et al. 2000).

Oxygen is necessary for the utilization of energy in a cell. However, the leakage associated with the reduction of oxygen during the oxidative phosphorylation cycle is a major source of free radicals. Peroxisomal metabolism and the metabolism of phagocytic leukocytes also produce ROS (Friedberg 1995). A number of xenobiotics and radiation can directly generate ROS (Klaassen 2001).

Free radicals possess unpaired electrons and are chemically very reactive. Through abstraction of hydrogen atoms or electrons, they can oxidize DNA, proteins, and lipids in cell membranes. DNA base modifications, abasic sites, deoxyribose damage, and single and double strand breaks are all induced following various forms of oxidative stress (Croteau and Bohr 1997). The most important of the ROS is the hydroxyl radical, which can react with purines to cause imidazole ring opening or with pyrimidines to cause ring contraction. These lesions may directly block DNA replication or may degrade with the formation of abasic sites. (Klaassen 2001). Oxidative DNA damage is thought to contribute to carcinogenesis, aging, and neurological degeneration (Croteau and Bohr 1997).

II.A.1.d. Depurination and Depyrimidination

Tautomeric shifts, deamination, alkylation, and oxidation all can indirectly lead to AP site formation. Such damage can enhance the hydrolysis rate of glycosidic linkages. In principle, any modification that converts the base to a better leaving group increases the rate of depurination /depyrimidination (Loeb and Preston 1986). Depurination is more frequent than depyrimidination as the glycosidic linkage is more stable in pyrimidine than in purine nucleosides (Lindahl and Karlstrom 1973). Other factors affecting the rate of N-glycosylic bond cleavage are pH and temperature. Once the loss

of the purine or pyrimidine occurs, the sugar at the AP site is a highly unstable cyclic carboxonium ion that undergoes hydrolysis to yield a diastereomeric mixture of 2-deoxy-α-D-ribose and 2-deoxy-β-D-ribose (Loeb and Preston 1986). As shown in Figure 4, the sugar anomers formed by the depurination/depyrimidination also exist in the much less favored open-ring aldehyde configuration about one percent of the time (Boturyn et al. 1999).

II.A.2. Environmental Damage.

Environmental (or exogenous) DNA damage can be caused by mutagenic agents that are either physical or chemical in nature. Many chemical and physical agents in the environment are capable of reacting with DNA. Substances potentially damaging to DNA include both man-made compounds and those of natural origin, such as food constituents and ultraviolet radiation. Some environmental agents enhance endogenous processes such as lipid peroxidation, while others have more direct effects.

II.A.2.a. Physical agents

Environmental damage caused by physical agents includes ionizing and UV radiation. Ionizing radiation can result in both direct and indirect damage to DNA. About 35% of such damage is attributable to direct ionization of components of the DNA. Indirect damage occurs when the radiation interacts with water and biological molecules to create reactive ions and free radicals (Friedberg 1995).

Ionizing radiation induces a variety of DNA lesions (Friedberg 1995). The rapidly dividing cell types (blood cell-forming areas of bone marrow, gastrointestinal tract lining) are the most affected by ionizing radiation and the severity of the effects depends upon the dose received (FDA 1979). In addition, ionizing radiation produces a

significant proportion of DNA damage as cluster DNA damage, where a combination of two or more damaged bases, AP sites, or single strand breaks are produced within a single radiation track (Sutherland et al. 2000). Significant inhibition of incision of an AP site by AP endonuclease occurs in the presence of an adjacent AP site which presents more challenges to the repair mechanisms in the cell (Cordonnier 2002).

UV light is electromagnetic radiation with wavelengths between 100 and 400 nm. It is non-ionizing and is significant as a mutagen mainly in wavelengths around 254 nm, the absorption peak of DNA bases. Sunlight is the major source of UV radiation. As opposed to the damage caused by ionizing radiation, most of UV light's effects result from direct interaction with DNA. UV light approaching 254 nm causes adjacent pyrimidines in DNA to form covalent bonds resulting in pyrimidine dimers. Although, pyrimidine dimers are the main premutation lesions in UV irradiated DNA, other photoproducts are also found, including the cross-linking of DNA to proteins, and strand breakage (Friedberg 1995).

Other sources also contribute to our radiation exposure. Among these are medical testing (diagnostic X-rays and other procedures), nuclear testing and power plants, and consumer products such as TV's, smoke detectors, airport X-rays. Taken together, the overall total average exposure from all sources is about 360 millirem/year; the major contributor of which is from natural sources such as radon gas (EPA 2000).

II.A.2.b. Chemical Agents

A variety of chemicals react directly with DNA and alter the structure and pairing properties of the bases. Alkylating agents are electrophiles that add alkyl groups to the nucleophilic centers on DNA. Alkylating agents can modify most nitrogen and oxygen

atoms in nucleosides by the addition of alkyl groups (Lawley 1966). Many carcinogens, chemical warfare agents, (e.g. mustards - chloroethylating compounds), and anti-cancer chemotherapeutic drugs are DNA alkylating agents. Alkylation often induces weakening of the N-glycosyl bond and by generating a better leaving group, promotes base release and AP site formation (Wilson and Barsky 2001). Many methylating and ethylating agents target guanine to yield N7 alkyldeoxyguanosine adducts with labile N-glycosidic bonds (Loeb and Preston 1986). Alkylation of the N^3 atoms of both purine nucleosides, or of the O^2 atoms of the pyrimidine nucleosides also induces formation of AP sites (Singer, Kroger, and Carrano 1978).

In addition, some alkylating agents are bifunctional (i.e., have two DNA-reactive groups). Cross linking occurs when the bifunctional alkylating agents react with nucleophilic centers of different nucleobases on the same strand or different strands in DNA (Friedberg 1995). Cross-linking agents and bulky adducts can cause significant distortion of the secondary structure of DNA. Interstrand DNA cross-links prevent DNA strand separation and represent complete blocks to replication. As such, many bifunctional alkylating agents like nitrogen mustard, mitomycin, platinum derivatives and psoralen derivatives have been used in cancer chemotherapy (Friedberg 1995).

Inactive chemicals can be metabolized to reactive metabolites that cause DNA damage. Electrophilic reactants can be generated by cytochrome P450 oxidation of xenobiotics. Polycyclic aromatic hydrocarbons, produced during combustion, and aflatoxins, produced by some fungi, are examples of such metabolically activated agents. Some chemicals, known as base analogs, structurally resemble purines and pyrimidines and may be incorporated into DNA in place of the normal bases during DNA replication.

Active oxygen species are also created during metabolism of a number of compounds and through the Fe (II)-catalyzed Fenton reaction (Timbrell 2000).

II.A.3 Base Excision Repair

DNA repair mechanisms are responsible for the maintenance of genetic integrity. The cell has two major defenses against mutations. If the damage is extensive, the cell can undergo apoptosis, or programmed cell death, which insures against the possibility of transforming mutations. Cells also have a range of repair processes that work to return damaged DNA to its error-free state. (Klaassen 2001). The mechanisms for coping with damage can be placed into three general categories: damage reversal, damage removal or excision, and damage tolerance. Damage reversal is the simplest type of repair where enzymatic action restores the normal structure without breaking the DNA backbone. DNA removal involves removing and replacing the damaged nucleotide or sequence containing the damaged nucleotide. Damage tolerance, is not truly repair, but rather a way of bypassing blocking lesions during replication (Friedberg 1985). While damage reversal and damage tolerance play important roles in allowing cells to deal with the consequences of damage, most DNA repair occurs when the damaged base is excised and subsequently replaced by the normal nucleotide (Friedberg 1995).

The BER pathway is considered one of the most important repair responses due to its capacity to remove a wide range of DNA adducts (Lindahl and Wood 1999). BER occurs when the damaged or inappropriate base is removed from its sugar linkage and replaced. BER corrects DNA modifications that arise either spontaneously or from attack by reactive chemicals. BER repairs mismatched or damaged bases that arise from replication errors or chemical modification, AP sites resulting from spontaneous or

mutagen-induced base release, and strand breaks which are the products of free radical attack (Klaassen 2001).

The DNA glycosylases catalyze the hydrolysis of the N-glycosylic bond linkage to the damaged base. Figure 5. shows an example of BER in which uracil glycosylase hydrolyzes the N-glycosylic bond between deoxyribose and the RNA base uracil generated by the deamination of cytosine. The enzyme recognizes the inappropriate base, uracil (Lewin 2000). This hydrolytic action removes the altered base and results in the formation of AP sites. The subsequent repair of the AP site is then initiated by apurinic / apyrimidinic (AP) endonuclease. The AP endonuclease cuts the damaged DNA strand at the AP site and creates a deoxyribose 5'-phosphate terminus which is removed by deoxyribophosphodiesterlyase and the repair is finished by sequential action of DNA polymerase and DNA ligase (Lewin 2000).

II.B. Mutations

Mutations are hereditary alterations of the DNA sequence. Premutagenic DNA lesions occur when the composition or structure of the DNA is modified leading to changes in sequence. The transition from a premutational lesion to a mutation often depends on which of two cellular processes occurs first, replication or repair. When damaged DNA is correctly repaired before replication, no mutation will result. When replication occurs before the lesion is repaired, attempted replication through the damage may result in a mutation. Some types of damage act as replication blocks so DNA synthesis does not proceed until the damage is repaired or bypassed via one of the tolerance mechanisms. Any one of several repair processes may be involved, and the usual outcome is that the normal chemical structure of the DNA is restored and the

original nucleotide sequence is recovered (Klaassen 2001). Mutations are important as they may have either deleterious or advantageous consequences to an organism. They are studied by geneticists in attempts to fix many genetic problems. They also are important as they are the major source of genetic variation, which fuels evolutionary change (Campbell 1996).

The relationship between DNA damage, repair, and various human diseases has been extensively studied. The response of an individual to a given initiatory event will vary according to a wide range of influences including toxicity, promotion, immune response, and individual sensitivity (Tardiff 1994). Mutations in genes that control normal cell proliferation can lead to cancer. Some cancers result from gene mutations which are inherited while others may be the result of inadequate DNA repair. Researchers have linked a reduction in DNA repair capacity to lung cancer (Klaassen 2001). Some genetic diseases are related to DNA repair and certain types of disorders are caused by defects in the genes that encode DNA repair enzymes. The increased susceptibility to mutation is shown in xeroderma pigmentosum patients that have an inherited disorder resulting in reduced DNA repair. People suffering from xeroderma pigmentosum are highly susceptible to malignant neoplasms caused by UV radiation (Cleaver and Kraemer 1995). DNA damage and inadequate repair have also been linked to other chronic diseases of aging (Atamna 2000). The existence of human diseases associated with defects in DNA repair illustrates the importance of repair as a genetic quality control mechanism (Lindahl and Wood 1987).

AP sites are both mutagenic and carcinogenic. The involvement of AP sites as promutagenic lesions varies with different mutagens (Loeb and Preston 1986). AP sites

promote misincorporation of nucleotides in vitro, and impede chain elongation during DNA replication and constitute a lethal lesion (Boturyn 1999). It has been demonstrated in vitro, that a single AP site can induce triplet repeat expansion, a phenomenon associated with neurological and neuromuscular diseases (Wilson 2001).

II.C. Importance of AP Sites and Quantification of Damage.

Given the role DNA damage plays in the makeup of a cell, it is important to be able to quantify any damage that occurs. Quantification can improve our knowledge of DNA damage and repair. In addition, detection of such DNA damage can contribute to understanding genotoxicity in human populations or in subpopulations after a particular exposure. Apurinic / apyrimidinic (AP) sites play a large role in DNA damage and repair. Cellular levels of AP sites are increased as a result of base modifications and their repair (Kubo et al. 1991). AP sites can have varying effects on replication and can lead to an accumulation of nicks or breaks in DNA strands. As shown in Figure 6, the 3' phosphodiester bonds associated with the open aldehyde forms are labile and can be hydrolyzed by an elimination reaction (Friedberg 1995).

AP sites are important intermediates in mutagenesis and carcinogenesis (Loeb and Preston 1986). It has been estimated that the spontaneous depurination rate per day is 10,000 bases per cell under normal physiological conditions (Lindahl and Nyberg 1972). More recent research using an in vivo ARP slot blot method determined that approximately 50,000-200,000 apurinic / apyrimidinic sites are present in each mammalian cell with the highest numbers being in the brain, colon, and heart. (Nakamura and Swenberg 1999). There have been many studies and methods designed to quantify AP sites (Kubo et al. 1992). Such techniques serve to elucidate questions concerning the

formation of AP sites, their repair, and help in the analysis of damage introduced in DNA by physical and chemical agents. (Talpaert-Borle' and Liuzzi 1983).

II.C.1. Alkali Elution

Alkali elution is another method used to detect AP sites in DNA and consists of converting primary lesions to more readily measured secondary forms. Lesions in alkali treated DNA are converted to strand breaks by treating the DNA with sodium hydroxide. The prevalence of strand breaks can then be determined by alkaline-CsCl sedimentation analysis (Brent et al. 1978). However, this method only determines intact AP sites. AP sites already associated with strand breaks are not detected as the assay is only based on the quantification of strand breaks that occur after chemical or enzymatic nicking of the phosphodiester bond 3' to the AP site. In addition, it is not always specific for oxidative lesions and may indicate DNA repair (Talpaert- Bole and Luizzi 1983).

II.C.2. Postlabeling Method

Weinfeld et al. described a postlabeling method for the detection of base damage. First, methoxyamine stabilizes the AP sites. Then AP sites are isolated and digested to dinucleosides. The dinucleosides containing the stabilized AP sites are then labeled in a reaction where the bacteriophage T4 polynucleotide kinase transfers a ^{32}P-labeled phosphate. The labeled dinucleosides are applied to a polyacrylamide / urea gel. After the gel has run and an autoradiogram has been obtained, the radiolabeled dinucleosides are excised and analyzed by HPLC. Such a method, by definition, does not require pre-

labeled DNA and only needs a very small amount of the DNA to detect AP sites (Weinfeld 1989).

II.C.3. Chemical Modification

AP sites have also been detected by modification with chemicals such as ^{14}C-methoxyamine (Talpaert-Borle' and Liuzzi 1983) and *O*-(nitrobenzyl)hydroxylamine (Kow, 1989). ^{14}C-Methoxyamine reacts with the aldehyde group present in the deoxyribose moiety after the nitrogenous base leaves. Unreacted ^{14}C-methoxyamine is separated from the ^{14}C-methoxyamine bound to the acid-insoluble DNA by washing with acid. The bound DNA is then counted with a liquid scintillation spectrometer (Talpaert-Borle' and Liuzzi 1983). AP sites can also react with *O*-(4-nitrobenzyl)hydroxylamine to produce *O*-(4-nitrobenzyl)hydroxylimmino Schiff bases. The resulting residues are then quantified by a monoclonal antibody raised against 5'-phosphodeoxyribosyl *O*-(4-nitrobenzyl)hydroxylamine as a way of determining the number of AP sites (Kow 1989).

II.C.4. Aldehyde Reactive Probe Method

Although there are several methods available to detect AP sites, many are very inconvenient or require the use of hazardous materials. The development of the aldehyde reactive probe (ARP) yielded a means of detection for AP sites that was not only very simple, but also rapid and accurate (Kubo 1992). In the presence of ARP, the equilibrium of AP sites between the ring-closed hemiacetal form and open chain aldehyde form shifts towards the latter so that potentially all abasic sites may react. The reaction in which ARP tags AP sites with biotin residues is shown in Figure 7.

The ARP penetrates the plasma membrane of the target cells and reacts with the AP sites to form an ARP-DNA stable adduct. The plates are washed with PBS-Tween to remove unreacted reagents and then treated with avidin-biotinylated horseradish peroxidase (Atamna 1999). This complex is used as the indicator enzyme to quantify the bound biotin tags colorimetrically by an assay similar to an ELISA (Kubo 1989). Such assays provide a sensitive and simple method to detect AP sites.

II.D. Synthetic Methodology

A publication by Ide et al. describes the synthesis for ARP. Modifications of this method were adopted for the synthesis of the iminoARP and the hydrazinoARP. The procedure involves *t*-BOC chemistry followed by the synthesis of an activated ester intermediate (Ide et al. 1993) (Figure 8). Similar to the Merrifield procedure used in the production of peptides, the aldehyde reacting amine group was first protected using *t*-BOC (Wade 1995). This step protects the amino moiety from self-condensation or from reacting with other reagents. The use of the dicyclohexylcarbodiimide (DCC) as a coupling agent results in an activated ester that can easily be attacked by biotin hydrazide in a nucleophilic substitution reaction. Trifluoroacetic acid cleaves the *t*-BOC protecting group and the resulting trifluoroacetic acid salt of ARP is then easily removed with the use of the base form of Dowex® resin.

III. Materials and Methods

III.A. Materials

All reagents used were purchased from Sigma-Aldrich Chemical Company and used as received. For column chromatography, grade 60 (200-400 mesh) silica gel was used. For preparative thin layer chromatography (TLC), 20 x 20 cm x 200 or 1000 microns silica plates were used (Analtech Company). For analytical TLC, 5 x 10 cm x 0.2 mm silica gel on aluminum plates were used.

DMSO-d_6 (99.99 %), D_2O (99.99 %), $CDCl_3$ (99.99 %) were all used as solvents for ^{1}H-NMR. For the final ARP products, 100.0 %-atom deuterium grade ^{1}H-NMR solvents were used.

III.B. Instrumentation

H^1 NMR spectra were obtained at 500 MHz on a Varian Inova 500 spectrometer (Varian, Palo Alto, CA). Chemical shifts were recorded in ppm relative to tetramethylsilane. FT processing was preformed using Mestre-C version 2.1.3 NMR processing software (Universidad de Santiago de Compostela, Spain)

Electrospray mass spectra were acquired by direct probe injection on a Finnigan LCQ^{DECA} mass spectrometer (ThermoQuest, San Jose). The spectrometer was operated in the positive ion mode and the mobile phase was 50 % methanol / 1 % acetic acid. Nitrogen was used as the auxiliary (40 psi) and sheath gases (80 psi). Argon was introduced to the collision cell at 2.5×10^{-3} mBar as the collision gas with a collision energy set to 25 V. Both high and low resolution MS was obtained as a service from the North Carolina State University Mass Spectrometry Facility.

III.C. Synthesis of ARP

III.C.1. *N*-(*tert*-Butoxycarbonyl)-*O*-(carboxymethyl)hydroxylamine (2)

O-(Carboxymethyl)hydroxylamine hemihydrochloride (**1**) (2.4 g, 22 mmol) and triethylamine (3.0 g, 30 mmol) were dissolved in 15 mL of DI water. Di-*tert*-butyl dicarbonate (5.9 g, 27 mmol) was dissolved in 15 mL of dioxane and was added to the solution. The reaction mixture was allowed to stir at room temperate for 3 days, after which 50 mL of DI water was added. The solution was extracted with ethyl acetate (3 x 70 mL). The water phase was cooled at 12 °C, and the pH was adjusted to 2 using 5 N HCl. The aqueous phase was extracted again with ethyl acetate (3 x 50 mL). The ethyl acetate phases were combined and washed with precooled 5% HCl (3 x 40 mL). The combined ethyl acetate layers were then washed with brine (3 x 40 mL), filtered through sodium sulfate, and evaporated to dryness. The product was recrystallized from hexane / ethyl acetate to give 2.5 g (60 %) of white crystals of **2**.

III.C.2. *N*-Hydroxysuccinimide Ester of *N*-(*tert*-Butoxycarbonyl)-*O*-(carboxymethyl)hydroxylamine (3)

N-(*tert*-Butoxycarbonyl)-*O*-(carboxymethyl)hydroxylamine (**2**) (2.3 g, 12 mmol) and *N*-hydroxysuccinimide (1.9 g, 17 mmol) were dissolved in 25 mL of dry acetonitrile (the acetonitrile was doubly distilled using P_2O_5 prior to the synthesis). Dicyclohexylcarbodiimide (3.3 g, 16 mmol) dissolved in 9 mL dry acetonitrile, was added to the mixture and the reaction stirred at room temperature for 24 hours. Precipitated dicyclohexylurea was removed by filtration and the filtrate was evaporated, leaving an oily residue. The residue was dissolved in 180 mL of hot carbon tetrachloride

and filtered hot. The filtrate was kept at 12 °C for five days and the resulting crystals were filtered giving 1.7 g (57 %) of **3**.

III.C.3. Biotin Hydrazide-*N*-(*tert*-Butoxycarbonyl)-*O*-(carboxymethyl)hydroxylamine Conjugate (4)

The *N*-hydroxysuccinimide ester of *N*-(*tert*-butoxycarbonyl)-*O*-(carboxymethyl)hydroxylamine (**3**) (0.1 g, 0.4 mmol) was dissolved in dimethylformamide (DMF) (6 mL) and biotin hydrazide (0.09 g, 0.4 mmol) was added. The solution was stirred overnight at room temperature and then was heated to 50 ° C in a mineral bath for one hour. The reaction was still cloudy, indicating that some biotin hydrazide remained unreacted. As a result, it was left at 50 °C for two days until the mixture became clear. The solvent was evaporated under reduced pressure using an oil pump and the residue was extracted with boiling ethyl acetate (3 x 100 mL). The extracts were combined and stored in the refrigerator for four days. The fine gel formed was filtered to yield 0.1 g (70 %) of **4**.

III.C.4. ARP (5)

The biotin hydrazide-*N*-(*tert*-butoxycarbonyl)-*O*-(carboxymethyl)hydroxylamine conjugate (**4**) (0.098 g, 0.229 mmol) was cooled at –10 °C and 1 mL of precooled trifluoroacetic acid was added with stirring. The solution was kept at –10 °C for 20 minutes and then at room temperature for 30 minutes. The trifluoroacetic acid was removed under a stream of argon gas. The resulting ARP trifluoroacetic acid salt was dissolved in 2.6 mL of DI water. Dowex® 1X-4 (100 mesh) resin was washed with several rinses of 5 % NaOH to convert it from the chloride form to the hydroxide form. The resin was added to the solution until the pH reached 7. The resin was removed by

filtration, washed with 1 mL of DI water, and then stirred for two hours in 10 mL of water. The resin was washed and stirred a second time to recover all of the ARP adsorbed to the resin. The filtrates were combined and the water was removed under reduced pressure leaving 0.06 g (79 %) white powder **5**. The entire synthetic scheme is shown in Figure 8.

III.D. Synthesis of ARP Components

In the event labeled ARP is needed, syntheses were devised for the production of ARP components, *O*-(carboxymethyl)hydroxylamine hemihydrochloride and the biotin hydrazide, based on commercially available [$^{13}C_2$]-bromoacetic acid and [$^{15}N_2$]-hydrazine. The procedures were verified using cold materials.

III.D.1. Biotin Hydrazide

D-Biotin-*p*-nitrophenyl ester (0.073 g, 2 mmol) was dissolved in 5 mL of dimethylformamide and hydrazine monohydrate (100 μL, 2 mmol) was added to the solution with stirring. The solution changed from a clear colorless to a bright yellow resulting from the release of *p*-nitrophenol. The reaction stirred overnight at room temperature and was heated to 50 °C during the last hour. The white precipitate was collected by filtration to yield 0.03 g (60 %) of product. The synthetic scheme is shown in Figure 9.

III.D.2 *O*-Carboxymethylhydroxylamine Hemihydrochloride

The *O*-(carboxymethyl)hydroxylamine hemihydrochloride was synthesized using the procedure of Anker and Clarke. To a solution of bromoacetic acid (0.612 g, 4.4 mmol) in 40 % aqueous sodium hydroxide (0.45 g, 4.4 mmol), acetoxime (0.33 g, 4 mmol) and sodium hydroxide (0.44 g, 4.4 mmol) were added with stirring. The solution

was kept below 15 °C during the addition of the alkali. The reaction was stirred at room temperature for 48 hours and the temperature was increased to 40 °C during the last hour of the reaction. The solution was then extracted three times with 0.5 mL of peroxide-free ether and then was precooled to 0 °C. While keeping the solution cooled below 15° C, 0.5 mL of concentrated HCl (6 mmol) was added to pH 1. The solution was saturated with sodium chloride and extracted six times with 1 mL of peroxide free ether. The fractions were combined and the ether was evaporated. To the acetone carboxymethoxime residue, 10 mL of DI water, 20 µL of hydroquinone, and 0.6 mL of conc. HCl were added with stirring. Acetone was removed from solution by distillation and the solution was concentrated to 1 mL. 2-Propanol (2 mL) was added, the solution was stored at -20 °C for 48 hours and then filtered yielding 0.1 g (27 %) of product. The synthetic scheme is shown in Figure 10.

III.E. Synthesis of IminoARP

III.E.1. 2-Iminobiotin Hydrazide-*N*-(*tert*-Butoxycarbonyl)-*O*-(carboxymethyl)hydroxylamine Conjugate (7)

The *N*-hydroxysuccinimide ester of *N*-(*tert*-butoxycarbonyl)-*O*-(carboxymethyl)hydroxylamine **(3)** (0.017 g, 0.058 mmol) synthesized as above, was dissolved in 2 mL of DMF and 2-iminobiotin hydrazide hydrochloride **(6)** (0.018 g, 0.058 mmol) was added with stirring. The solution stirred at room temperature for 48 hours and then the temperature was raised to 50 °C for an additional hour. DMF was evaporated under oil pump vacuum and the oily residue was extracted (3 x 4 mL) with boiling 2-propanol. After 48 hours, crystals in the 2-propanol extract were centrifuged and the mother liquor was slowly and carefully drawn off with a pipette. The mother

liquor was concentrated to 0.2 mL, cooled to 12 °C, and additional product was recovered.

III.E.2. IminoARP (8)

The product (**7**) was cooled for 24 hours at -20 °C and 2 mL of precooled trifluoroacetic acid was added with stirring. The mixture was kept at 0 °C for 20 minutes and then stirred at room temperature for 30 minutes. The trifluoroacetic acid was removed under a stream of argon gas. The remaining salt was dissolved in 2 mL of DI water. Dowex® 1X-4 resin, which had been washed and converted to the OH^- form, was added until the pH was in the 3-4 range. The resin was filtered and rinsed with DI water. To remove product adsorbed to the resin, the resin was stirred in 3 mL of DI water for 4 hours, filtered and rinsed with additional DI water. This procedure was repeated 3 times. The DI water fractions were combined and the water was removed under reduced pressure yielding 0.013 g (62 % over all yield) of **8**. Figure 11 shows the synthesis.

III.F. HydrazinoARP

III.F.1. Attempted Syntheses of Hydrazinoacetic Acid

A number of approaches to the attempted syntheses of hydrazinoacetic acid were attempted and are presented in Figure 12.

III.F.1.a. Approach 1

Bromoacetic acid (0.527 g, 3.77 mmol) was dissolved in 5 mL of DMF in a round bottom flask. Using a graduated pipette, 0.2 mL of hydrazine monohydrate (0.179 g, 3.7 mmol, d = 1.032 g/mL) was added to the solution with stirring. The solution initially turned cloudy but after 24 hours became clear. The DMF was evaporated leaving a light

brownish-orange residue. Ethyl alcohol was used to precipitate the residue and the crystals were collected by filtration. ^{1}H NMR showed only hydrazine to be present.

III.F.1.b. Approach 2

The above reaction was repeated following conversion of bromoacetic acid to the sodium salt. Sodium bicarbonate was used to protect the carboxyl group from reacting with hydrazine.

III.F.1.c. Approach 3

Approach 1 was repeated using tertiary butyl carbazate, but hydrazinoacetic acid was not formed in this reaction either. Approach 3 was also repeated after converting bromoacetic acid to a salt. In addition, bromoethyl acetate was also used in place of bromoacetic acid. However, in these syntheses as well, only hydrazine could be identified by ^{1}H NMR.

III.F.1.d. Approach 4

The synthesis of hydrazinoacetic acid was attempted by preparing ethyl hydrazineacetate followed by hydrolysis using the procedure from A. Carmi, G. Pollak, and H. Yellin. Bromoacetic acid (0.562 g, 4.02 mmol) was placed in a 25 mL round bottom flask and dissolved in 5 mL DMF and 0.3 mL of hydrazine monohydrate (3.01g, 6.00 mmol) was added. The solution was stirred for 48 hours at room temperature. Sodium hydroxide (0.55 g, 11 mmol) was added and the solvent was evaporated. The residue was treated with 4 ml of ethanol HCl 30 % w/v, (3.3 mL of 36 % HCl was diluted to 4 mL with ethanol) turning the solution cloudy. The solution was refluxed for 1.5 hours in a mineral oil bath at 100 °C and then saturated with HCl gas for 45 minutes. After saturation, 10 mL of absolute ethyl alcohol was added and the reaction refluxed

overnight at 105 °C and filtered hot. The filtrate was kept refrigerated for two days. The resulting crystals were refluxed for 3 hours at 110° C in 3.5 mL of water and 0.15 g of Dowex® 50Wx8-200 (acid form). The filtered solution was passed through a column with Duolite® A-7 (base form) and then concentrated to 0.5 mL. The concentrated solution was added dropwise into 2.5 mL of absolute ethyl alcohol. The ^{1}H NMR spectrum showed only the presence of water and solvent.

III.F.1.e. Approach 5

The synthesis of hydrazinoacetic acid was also attempted using glyoxylic acid. To a 25 mL round bottom flask containing glyoxylic acid (0.20 g, 2.7 mmol), was added a solution of hydrazine monohydrate (0.135 g, 2.7 mmol) dissolved in 7 mL of ethanol. The solution was stirred for 6 hours. However, no product was observed by ^{1}H NMR.

III.F.1.f. Approach 6

Glycine was also used as a starting point to synthesize hydrazinoacetic acid. In 18 mL of DI water, glycine (1.0 g, 13 mmol) and potassium cyanate (1.1 g, 13 mmol) were dissolved with stirring. Concentrated HCl (1.3 mL, 13 mmol) was added to the solution and the solution stirred at 60 °C overnight. The solution was cooled to room temperature and the insoluble material filtered off. The ice-cooled solution was then acidified by the addition of 5.4 mL of concentrated HCl and the solvent evaporated. No product was observed by ^{1}H NMR.

III.F.2. HydrazinoARP

This successful approach was based on coupling *N*-*t*-Boc-protected hydrazine with the *N*-hydroxysuccinimide ester of bromoacetic acid (Figure 13). Prior to the synthesis, acetonitrile was freshly distilled using P_2O_5.

III.F.2.a. *N*-Hydroxysuccinimide Ester of Bromoacetic Acid (10)

Bromoacetic acid (0.54 g, 3.8 mmol) was dissolved in 10 mL of the dry acetonitrile and *N*-hydroxysuccinimide (**9**) (0.5 g, 4.3 mmol) was added with stirring. Next, DCC (0.88 g, 4.4 mmol) was dissolved in 3 mL of dry acetonitrile and added to the solution. Dicyclohexylurea began to precipitate. The reaction was stirred at room temperature overnight. Dicyclohexylurea was filtered off and the solvent was evaporated to give 0.7 g (77 %) of **10**.

III.F.2.b. *N*-Hydroxysuccinimide Ester of N^{l}-Carboxymethyl-tertiary Butyl Carbazate (11)

The *N*-hydroxysuccinimide ester of bromoacetic acid (**10**) (0.38 g, 1.84 mmol) was added to a solution of tertiary butyl carbazate (0.21 g, 1.6 mmol) and triethylamine (0.16 g, 1.6 mmol) in 10mL of methylene chloride. The solution stirred for 4 hours and was washed with 5 % HCl (3 x 6 mL) and then with brine (3 x 6 mL). The methylene chloride fraction was dried over sodium sulfate and the solvent was evaporated under reduced pressure. The residue was dissolved in 3 x 20 mL of boiling carbon tetrachloride and filtered while hot. After 2 days in the refrigerator, 0.07 g (16 %) of crystalline product (**11**) was collected by filtration.

III.F.2.c. Biotin Hydrazide - N^{l}-Carboxymethyl-tertiary Butyl Carbazate Conjugate (12)

The *N*-hydroxysuccinimide ester (**11**) (0.021 g, 0.073 mmol) was dissolved in 3 mL of DMSO and biotin hydrazide (0.018 g, 0.073 mmol) was added with stirring. The reaction was stirred for 3 days during which biotin hydrazide began to dissolve as the reaction progressed. Finally, the reaction was heated at 50 °C for 2 hours. The DMF was

removed under reduced pressure and the residue was extracted three times with 4 ml of boiling ethyl acetate. The extracts were combined and were placed at -20 °C for 2 days and 0.020 g (64 %) of **12** was collected by filtration.

III.F.2.d. HydrazinoARP (13)

Biotin hydrazide - **11** conjugate (**12**) (0.020 g, 0.046mmol) was cooled at 0 °C and then dissolved in 1 mL of precooled trifluoroacetic acid. The reaction was stirred at 0 °C for 20 min. and then at room temperature for 30 min. The trifluoroacetic acid was then removed under a stream of argon gas. The residue was dissolved in 5 mL of DI water and Dowex® 1X-4 resin (converted to the OH^- form) was added to the solution until the pH reached 7.0. The resin was filtered and rinsed with 5 mL of DI water. The resin was then stirred in 5 mL DI water for three hours, filtered, and rinsed with additional DI water. This was repeated twice to recover additional product. The fractions were combined and the water was evaporated under reduced pressure leaving 0.010 g (70 %) of hydrazinoARP (**13**). The hydrazinoARP synthesis scheme is presented in Figure 13.

III.G. 16-Hydroxyhexadecanoic Acid Probe

III.G.1. *N*-Hydroxysuccinimide Ester of 16-Hydroxyhexadecanoic Acid (15)

16-Hydroxyhexadecanoic acid (**14**) (0.27 g, 1.0 mmol) and *N*-hydroxysuccinimde (0.13 g, 1.1 mmol) were dissolved in 15 mL of THF, doubly distilled, first from calcium hydride and then from lithium aluminum hydride. DCC (0.23 g, 1.1 mmol) was dissolved in THF and added to the solution with stirring. The reaction was stirred at room temperature for 2 days. The precipitated dicyclohexylurea was filtered off and the solvent was evaporated under reduced pressure. The residue was then dissolved in

chloroform and chromatographed on a preparative TLC plate eluted with $CHCl_3$/MeOH 24:1 v/v. Four bands were obtained, and the first two bands eluted were determined to contain product (reaction shown in Figure 14).

III.G.2. Synthesis of *N*-Phthalimido Derivative of the *N*-Hydroxysuccinimide Ester of 16-Hydroxyhexadecanoic Acid

To 10 mL of fresh doubly distilled THF, **15** (0.13 g, 0.33 mmol) was added. Triphenyl phosphine (0.088 g, 0.33 mmol), *N*-hydroxyphthalimide (0.054 g, 0.33 mmol), and diethylazodicarboxylate (0.058 g, 0.33 mmol) were then added to the solution with stirring. After stirring at room temperature for 14 hours, THF was evaporated under reduced pressure. The residue was dissolved in 4 mL of dichloromethane and extracted with 2 mL of water. The organic layer was dried over sodium sulfate and applied to a preparative TLC plate. The plate was developed using 99:1 (v/v) chloroform / methanol and yielded 5 bands. However, analysis of the bands by ^{1}H NMR and mass spectrometry failed to detect the expected product (reaction shown in Figure 15).

III.G.3. Biotin Hydrazide-16-Hydroxyhexadecanoic Acid Conjugate (16)

The *N*-hydroxysuccinimide ester of 16-hydroxyhexadecanoic acid **(15)** (0.040 g, 0.11 mmol) was dissolved in DMF (2mL). Biotin hydrazide (0.026 g, 0.10 mmol) was added with stirring. The reaction was stirred overnight at room temperature and then at 50 °C for 2 hours. Biotin hydrazide dissolved as the reaction proceeded. The DMF was removed under reduced pressure and the residue extracted with 3 x 4 mL of boiling ethyl acetate. The ethyl acetate fractions were combined and stored at -20 °C for 2 days. Crystals were collected by filtration yielding 0.030 g (59 %) of **16**. The synthetic scheme is presented in Figure 16.

III.G.3. *N*-Phthalimido Derivative of Biotin Hydrazide-16-Hydroxyhexadecanoic Acid Conjugate (17)

Biotin hydrazide conjugate of 16-hydroxyhexadecanoic acid (**16**) (0.012 g, 0.025 mmol) was dissolved in 5 mL of DMSO freshly distilled from calcium hydride. *N*-Hydroxyphthalimide (0.004 g, 0.025 mmol) was added, followed by DCC (0.005g, 0.025 mmol) dissolved in 2 mL of dry acetonitrile (distilled off P_2O_5). The reaction mixture stirred for 24 hours at room temperature and the temperature was then increased to 80 °C for an additional hour. The DMSO was removed and the product was dissolved in 4 mL of DMF. The precipitate was filtered out and the DMF solution (filtrate) was evaporated under reduced pressure to afford a light yellow residue. The residue was extracted with boiling ethyl acetate (3 x 4 mL). The extracts were combined and stored in the refrigerator for 2 days. The product was collected by filtration to yield 5 mg (31 %) of **17** (Figure 17).

III.G.4. Attempted Synthesis of 16-Hydroxyhexadecanoic Acid ARP

The *N*-hydroxyphthalimido derivative (**17**) (0.0033g, 0.0050mmol) was dissolved in 5 mL of DMF and cooled to 0 °C. To the solution, 1 mL of 0.5 ug/mL of hydrazine monohydrate was added dropwise over ten minutes. The solution stirred at room temperature for 50 minutes and then 3 mL of diethyl ether was added. The solution was kept in the refrigerator overnight and then evaporated under reduced pressure. ^{1}H NMR analysis showed that the desired product was not present but hydrazide of *O*-(16-carboxyhexadecanoic) hydroxylamine had formed through displacement of biotin hydrazide (Figure 18).

IV. Results

All the compounds synthesized were characterized by ^{1}H NMR. Proton chemical shifts are reported in parts per million (δ) relative to TMS. Products were further characterized by mass spectrometry. Structures of previously unreported compounds were also determined by high resolution mass spectrometry.

IV.A. Synthesis of ARP

IV.A.1. *N*-(*tert*-Butoxycarbonyl)-*O*-(carboxymethyl)hydroxylamine (2)

(DMSO-d_6) δ1.37 (s, 9H, t-BuH), 4.21 (s, 2H, CH_2), 10.11 (br s, 1H, NH), Figure 19.

IV.A.2. *N*-Hydroxysuccinimide Ester of *N*-(*tert*-Butoxycarbonyl)-O-(carboxymethyl)hydroxylamine (3)

(DMSO-d_6) δ 1.37 (s, 9H, t-BuH), 2.82 (s, 4H, CH_2-CH_2), 4.81 (s, 2H, CH_2O), 10.37 (br s,1H, NH), Figure 20.

IV.A.3. Biotin hydrazide-(*tert*-Butoxycarbonyl)-*O*-(carboxymethyl)hydroxylamine conjugate (4)

(DMSO-d_6) δ 1.2-1.7 (m, 6H, H_9, H_{10}, H_{11}), 1.41 (s, 9H, t-BuH), 2.11 (t, J = 7 Hz, 2H, H_{12}), 2.58 (d, J = 12 Hz, 1H, H_{7a}), 2.81 (dd, J = 5, 12 Hz, 1H, H_{7b}), 3.10, (m, 1H, H_5), 4.12 (m, 1H, H_4), 4.25 (s, 2H, H_{17}), 4.29 (M, 1H, H_8), 6.34 (br s, 1H, H_{14}), 6.40 (brs, 1H, H_{15}), 9.77 (br s, 2H, H_1, H_3), 10.17 (br s, 1H, H_{19}), Figure 21.

IV.A.4. ARP (5)

(DMSO-d_6) δ 1.2-1.7 (m, 6H, H_9, H_{10}, H_{11}), 2.11 (t, J = 7.2 Hz, 2H, H_{12}), 2.55 (d, J = 12.3 Hz, 1 H, H_{7a}), 2.79 (dd, J = 5, 12 Hz, 1H, H_{7b}), 3.07 (m, 1H, H_5), 4.02 (m, 1H,

H_4), 4.12 (s, 2H, H_{17}), 4.30 (m, 1H, H_8), 6.34 (br s, 1H, H_{19}), 6.40 (brs, 2H, H_{14}, H_{15}), 9.77 (br s, 2H, H_1, H_3), Figure 22.

MH+ calculated 332.1392, found 332.56, Figure 23.

IV.B. ARP components

IV.B.1. Biotin hydrazide

(D_2O) δ 1.2-1.7 (m, 6H, H_9, H_{10}, H_{11}), 2.11 (t, J = 7.3 Hz, 2H, H_{12}), 2.55 (d, J = 12.7 Hz, 1 H, H_{7a}), 2.76 (dd, J = 5.0, 12.0 Hz, 1H, H_{7b}), 3.07 (m, 1H, H_5), 4.14 (m, 1H, H_4), 4.12 (s, 2H, H_{17}), 4.32 (M, 1H, H_8), Figure 24.

IV.B.2. *O*-(Carboxymethyl)hydroxylamine

(DMSO-d_6) δ 4.56 (s, 2H, CH_2), Figure 25, Figure 26.

IV.C. IminoARP

IV.C. 1. 2-Iminobiotin Hydrazide Hydrochloride-*N*-(*tert*-Butoxycarbonyl)-*O*-(carboxymethyl)hydroxylamine Conjugate (7)

(DMSO-d_6) δ1.2-1.7 (m 6H, H_9, H_{10}, H_{11}), 1.41 (s, 9H, t-BuH), 2.11 (t, J = 7.3 Hz, 2H, H_{12}), 2.78 (d, J = 13.58 Hz, 1H, H_{7a}), 2.89 (dd, J = 4.7, 12.5 Hz, 1H, H_{7b}), 3.21 (m, 1H, H_5), 4.25 (s, 2H, H_{17}), 4.43 (m, 1H, H_4), 4.62 (m, 1H, H_8), 8.01 and 8.18 (br s, 2H, H_{14}, H_{15}), 9.80 (br s, 2H, H_1, H_3), 10.19 (br s, 1H, H_{19}), Figure 27.

IV.C. 2. IminoARP (8)

(D_2O-d_6) δ1.2-1.7 (m 6H, H_9, H_{10}, H_{11}), 2.22 (t, J = 7.2 Hz, 2H, H_{12}), 2.75 (d, J = 13.8 Hz, 1H, H_{7a}), 2.90 (dd, J = 4.6, 13 Hz, 1H, H_{7b}), 3.27 (m, 1H, H_5), 4.20 (s, 2H, H_{17}), 4.50 (m, 1H, H_4), 4.64 (m, 1H, H_8), Figure 28.

(MH^+) calcd 331.412, found 331.51, Figure 29.

IV.D. HydrazinoARP

IV.D.1. *N*-Hydroxysuccinimide Ester of Bromoacetic Acid (10)

(DMSO-d_6) δ 2.82 (s 4H, CH_2-CH_2), 4.62 (s, 2H, CH_2.Br), Figure 30.

IV.D.2. *N*-Hydroxysuccinimide Ester of N^1-Carboxymethyl-tertiary Butyl Carbazate (11)

(DMSO-d_6) δ 1.38 (m, 9H, t-BuH), 2.60 (s, 4H, CH_2-CH_2), 4.51 (s, 2H, CH_2), 8.86 (br s, 1H, NH), 9.78 (br s, 1H, NH), Figure 31.

MH+ calculated 288.27.

FAB

MNa^+ calculated 310.273, found MNa^+ (FAB) 310.273, Figure 32.

ES

MNa^+ found 310.0, Figure 33.

IV.D.3. Biotin Hydrazide-N^1-Carboxymethyl-tertiary Butyl Carbazate Conjugate (12)

(DMSO-d_6) δ1.2-1.7 (m 6H, H_9, H_{10}, H_{11}), 1.41(s, 9H, t-BuH), 1.97 (t, J = 7.3 Hz, 2H, H_{12}), 2.54 (d, J = 11.9 Hz, 1H, H_{7a}), 2.78 (dd, J = 5.3, 12.5 Hz, 1H, H_{7b}), 3.07 (m, 1H, H_5), 4.28 (m, 1H, H_4), 4.10 (m, 1H, H_8), 4.51 (s, 2H, H_{17}), 6.33 and 6.40 (br s, 2H, H_{14}, H_{15}), 8.86 (br s, 1H, H_{18}), 8.91 (br s, 2H, H_1, H_3), 9.79 (br s, 1H, H_{19}), Figure 34.

IV.D.4. HydrazinoARP (13)

(DMSO-d_6) δ 1.2-1.7 (m, 6H, H_9, H_{10}, H_{11}), 1.99 (t, J = 7.4 Hz, 2H, H_{12}), 2.54 (d, J = 11.7 Hz, 1H, H_{7a}), 2.78 (dd, J = 5.4, 12.0 Hz, 1H, H_{7b}), 3.07 (m, 1H, H_5), 4.11 (m, 3H, H_4, H_{17}), 4.29 (m, 1H, H_8), 6.33 (br s, 1H, H_{14}), 6.40 (brs, 1H, H_{15}), 8.90 (br s, 2H, H_1, H_3), Figure 35.

IV.E. 16-Hydroxyhexadecanoic Acid ARP

IV.E.1 16-Hydroxyhexadecanoic Acid (14)

($CDCl_3$-d_6) δ 1.2-1.4 (m, 18H, $C5H_2$-$C13H_2$), 1.57 (m, 4H, $\underline{CH_2CH_2}CH_2COOH$), 1.64 (m, 4H, $\underline{CH_2CH_2}CH_2OH$), 2.35 (t, J = 7.7 Hz, $\underline{CH_2}COOH$), 3.65 (t, J = 6.2 Hz, 1H, $\underline{CH_2}OH$), Figure 36.

IV.E.2. *N*-Hydroxysuccinimide Ester of 16-Hydroxyhexadecanoic Acid (15)

($CDCl_3$-d_6) δ 1.2-1.4 (m, 18H, $C5H_2$-$C13H_2$), 1.54 (m, 4H, $\underline{CH_2CH_2}CH_2COOR$), 1.73 (m, 4H, $\underline{CH_2CH_2}CH_2OH$), 2.35 (t, J = 7.2 Hz, $\underline{CH_2}COOR$), 2.84 (s, 4H, $O{=}C\underline{CH_2CH_2}C{=}O$), 3.65 (t, J = 6.2 Hz, 1H, $\underline{CH_2}OH$), Figure 37.

IV.E.3. Biotin Hydrazide-2 Conjugate (16)

(DMSO-d_6) δ 1.2-1.8 (m, 32H, $C9H_2$-$C11H_2$, $(CH_2)_{18}$-$(CH_2)_{30}$), 2.07 (t, J = 7.6 Hz, 2H, $(CH_2)_{12}$), 2.57 (d, J = 11.7 Hz, 1H, H_{7a}), 2.61 (t, J = 7.2 Hz, 2H, $(CH_2)_{17}$), 2.73 (m, 3H, H_{7b}, $(CH_2)_{31}$), 3.08 (m, 1H, $(CH)_5$), 4.12 (m, 1H, $(CH)_4$), 4.29 (m, 1H, $(CH)_8$), 6.34 (br s, 1H, N14-H), 6.41 (brs, 1H, N15-H), 9.61 (br s, 2H, N1-H, N3-H), Figure 38.

IV.E.4. *N*-Phthalimido Derivative of 3 (17)

(DMSO-d_6) δ 1.2-1.8 (m, 32H, $C9H_2$-$C11H_2$, $C18H_2$-$C30H_2$), 2.07 (t, J = 7.6 Hz, 4H, $(CH_2)_{12}$, $(CH_2)_{17}$), 2.50 (d, J = 12.5, 1H, H_{7a}), 2.79 (dd, J = 4.7,12.8, H_{7b}), 3.07 (m, 1H, $(CH)_5$), 4.11 (m, 1H, $(CH)_4$), 4.29 (M, 1H, $(CH)_8$), 6.33 (br s, 1H, N14-H), 6.39 (brs, 1H, N15-H), 7.94 (s, 4H, $(CH)_{36\text{-}39}$), 9.61 (br s, 2H, N1-H, (N3-H), Figure 39.

V. Discussion

In this project ARP, iminoARP, and hydrazinoARP were successfully synthesized and their structures were confirmed by NMR and mass spectrometry. The syntheses were reproducible and resulted in adequate yields. ARP and iminoARP were synthesized reproducibly on different scales. For most of the reaction steps, the yields ranged from 60 to 90 %. ARP had the highest overall yield and hydrazinoARP resulted in the lowest yield, but since the route is newly developed it is likely that yields may be further optimized. The steps preceding the coupling reaction with biotin hydrazide (or iminobiotin hydrazide) (Figures 8, 10, 13) resulted in the lowest yields. The enantiomeric synthons, biotin hydrazide and iminobiotin hydrazide, are commercially available (Aldrich) and reagent cost is not a limiting factor. Reactions following the biotin hydrazide coupling step all resulted in adequate yields. Therefore, the synthesis of the reactive probes is convenient and relatively inexpensive.

In the final stage of the ARP and hydrazinoARP syntheses, the base form of Dowex® resin was used to bring solutions to a pH of 7. This was an important step and the amount of contaminants on the NMR seemed to correlate with the accuracy of the pH. The products highest in purity were obtained when a pH meter was used to monitor the solution, rather than the pH strips, which only gave a pH range. Iminium salts are unstable at neutral and basic pHs. In addition, at higher pHs, protonation on the amine group may inhibit the reaction with the AP sites and decrease the usefulness of the iminobiotin derivative as an abasic site probe. Therefore, during the synthesis of the iminoARP, the compound was never brought above pH of 3-4. Even in a protonated form in 3-4 pH range, the adsorbtion of the iminoARP to the resin was not a problem and

a sufficient yield could be obtained by subsequent washings of the resin. When iminoARP was brought to a neutral pH, the synthesis did not work.

As described in the methods section, several approaches were attempted before achieving the synthesis of hydrazinoacetic acid. In the unsuccessful reactions, the reactivity of hydrazine resulted in the predominance of side reactions. Furthermore, the few reported routes to hydrazinoacetic acid were not reproducible. The probe was synthesized by first protecting bromoacetic acid as a N-hydroxysuccinimide ester to avoid formation of the hydrazide. The ester was then reacted with tertiary butyl carbazate to produce the *N*-hydroxysuccinimide ester of tertiary butyloxycarbonyl hydrazide. Once this compound was successfully synthesized, the procedure from Ide et al. could be used to complete the synthesis of the probe.

Synthesis of the 16-hydroxyhexadecanoic reactive probe has not been completed. *N*-hydroxysuccinimide was used to protect the carboxy group of 16-hydroxyhexadecanoic acid. However, at the next stage of the synthesis, protection of the 16-hydroxy group by *N*-hydroxyphthalimide could not be accomplished with either the DCC or DEAD coupling procedures. The activated ester formed as a result of the addition of *N*-hydroxysuccinimide to the carboxy group **(15)** was apparently too strong an electrophile and the addition of the *N*-hydroxyphthalimide only resulted in a transesterification reaction rather than the synthesis of an ether. Since amide bonds are less reactive than esters, the order of probe assembly was altered to couple the *N*-hydroxysuccinimide ester with biotin hydrazide, creating an amide bond (Figure 16). A DCC coupling reaction resulted in the protection of the alcohol functional group with *N*-hydroxyphthalimide. Building upon the approach by Krotz et al., hydrazine was used in

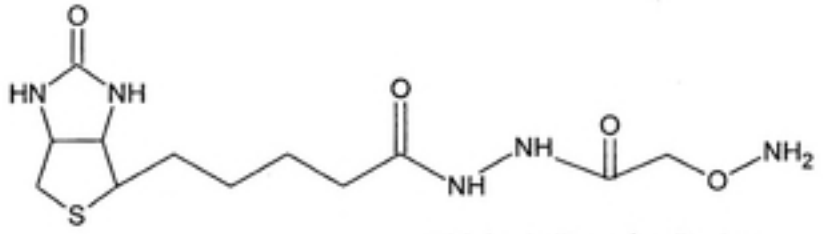
a reaction to convert the *N*-hydroxyphthalimide ether to a hydroxylamine derivative capable of reacting with aldehydes. However, this final stage of the synthesis has not yet been completed. The hydrazine split the hydrazido linkage between the 16-hydroxyhexadecanoyl tether and biotin resulting in the displacement of the biotin group instead of reacting at the carbonyl carbons of *N*-hydroxyphthalimide (Figure 17). Alternative procedures for removing phthalic acid would be to use weaker bases.

Procedures were also developed to prepare some of the reagents needed in the ARP synthesis for labeled internal standards. The synthesis of biotin hydrazide and *O*-(carboxymethyl)hydroxylamine both involved reagents that are commercially available in the labeled form, [$^{15}N_2$]-hydrazine and [$^{13}C_2$]-bromoacetic acid. Because of the expense associated with labeled materials, moderate to high yields should be obtained. Biotin hydrazide was synthesized with a high yield and because it is introduced near the end of the synthetic scheme of ARP, the overall recovery of labeled product should be high. However, very low yields were obtained in the *O*-(carboxymethyl)hydroxyl-amine hemihydrochloride synthesis, only about 27 %. In addition, an overall yield of only about 20 % has been achieved in the ARP reaction. Since *O*-(carboxymethyl)hydroxyl-amine hemihydrochloride is the first reagent introduced in the synthesis of ARP, use of this compound in the route to labeled ARP may not be cost effective.

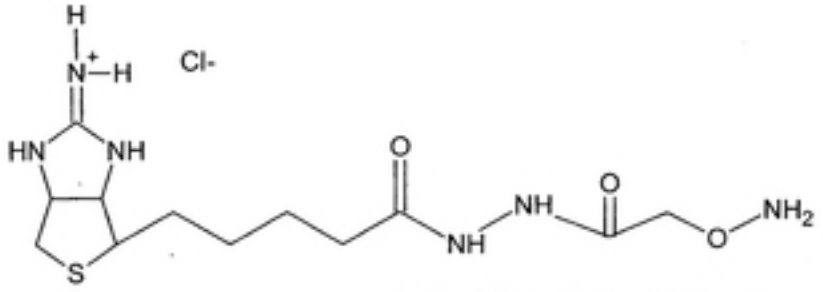
VI. Conclusions

This work resulted in the synthesis of new compounds. These compounds could be important in detecting AP sites and could have wide public health implications. They provide a way to easily, sensitively, and safely detect AP sites from DNA. AP sites can then be exposed to various environmental agents and then quantified in cells using the aldehyde reactive probes synthesized, and the number of sites can be compared to those in control cells. Using the new different probes AP sites can be more conveniently quantified.

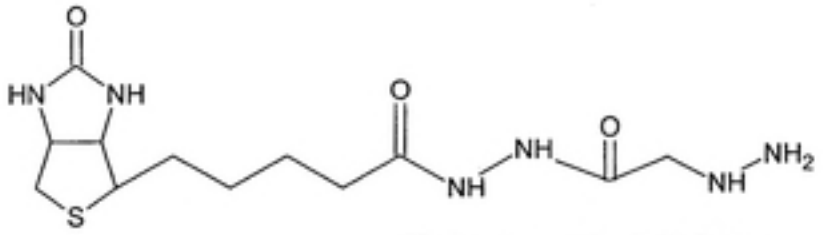
This research results in additional areas of study that can be further investigated. Research is needed in determining the effectiveness of the novel ARPs. Only the ARP and iminoARP synthesized in this project have been tested in quantification assays, but the results do show potential and indicate their effectiveness (Nakamura, unpublished data). In addition, while the 16-hydroxyhexadecanoic probe was not successfully completed, the synthetic pathway has been largely developed; only the final step needs further work-up. Finally, other ways to introduce labeled materials into the synthesis of ARP can be developed, and additional ARP reactive probes can be made.



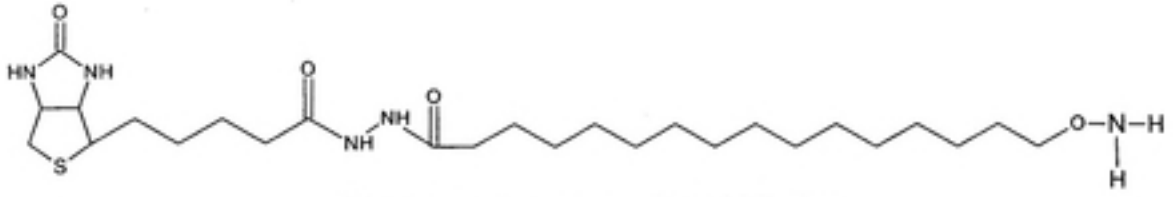
Aldehyde Reactive Probe



Iminobiotin Hydrazide Probe



Hydrazinoacetic Acid Probe



16-Hydroxyhexadecanoic Acid Probe

Figure 1. AP Probes.

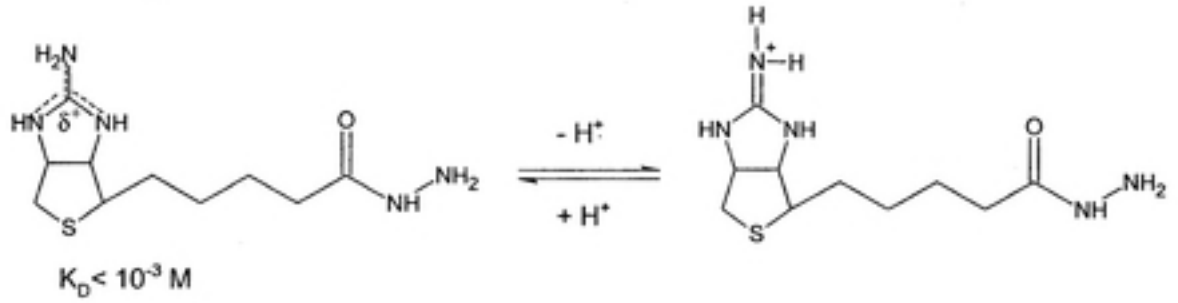


Figure 2. Ionization characteristics of 2-iminobiotin hydrazide.

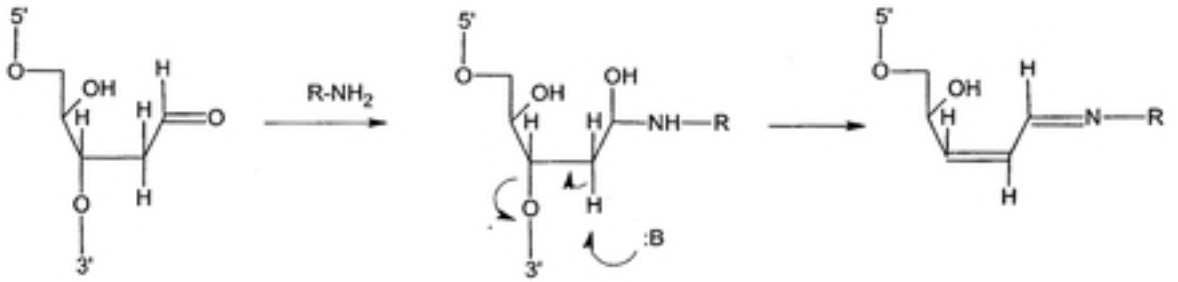


Figure 3. Talpaert-Borle' and Lizzi hypothetical pathway for the 3' cleavage of DNA containing AP sites after the reaction with an aldehyde reagent R-NH_2

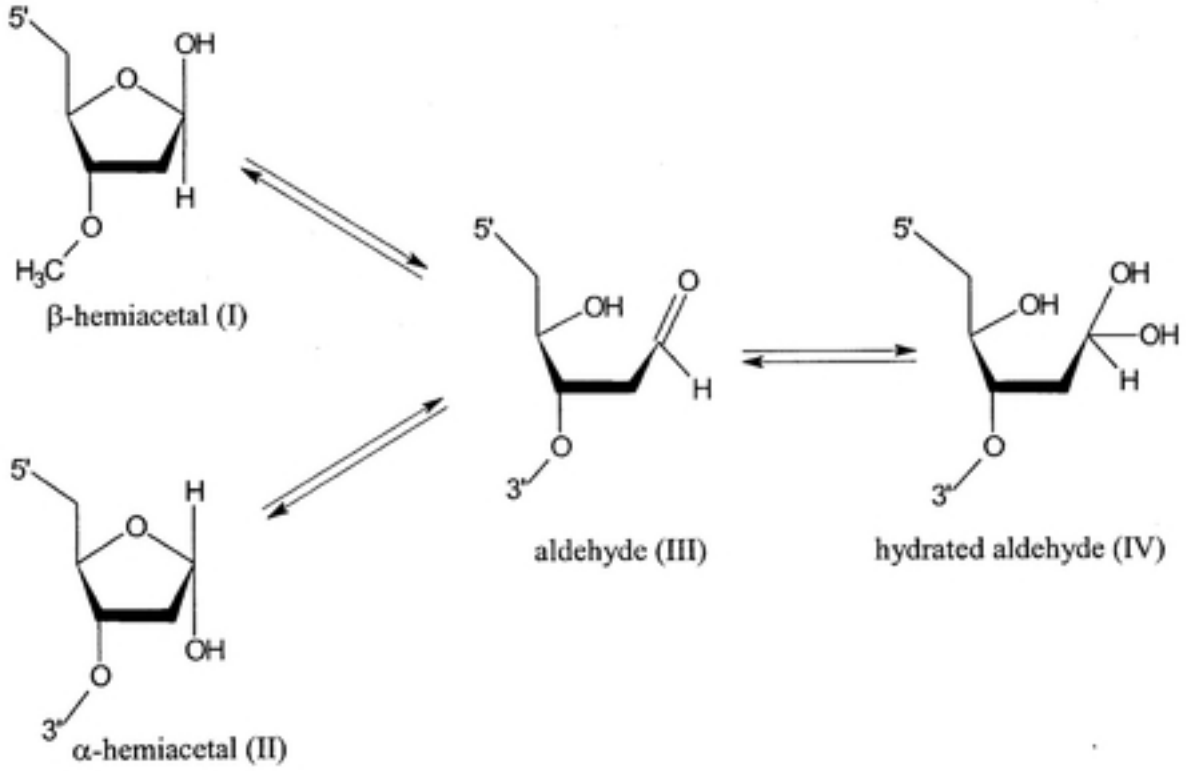


Figure 4. The cleavage of the glycosidic bond in DNA leads to an abasic site that exists in equilibrium between a closed ring and open aldehyde form.

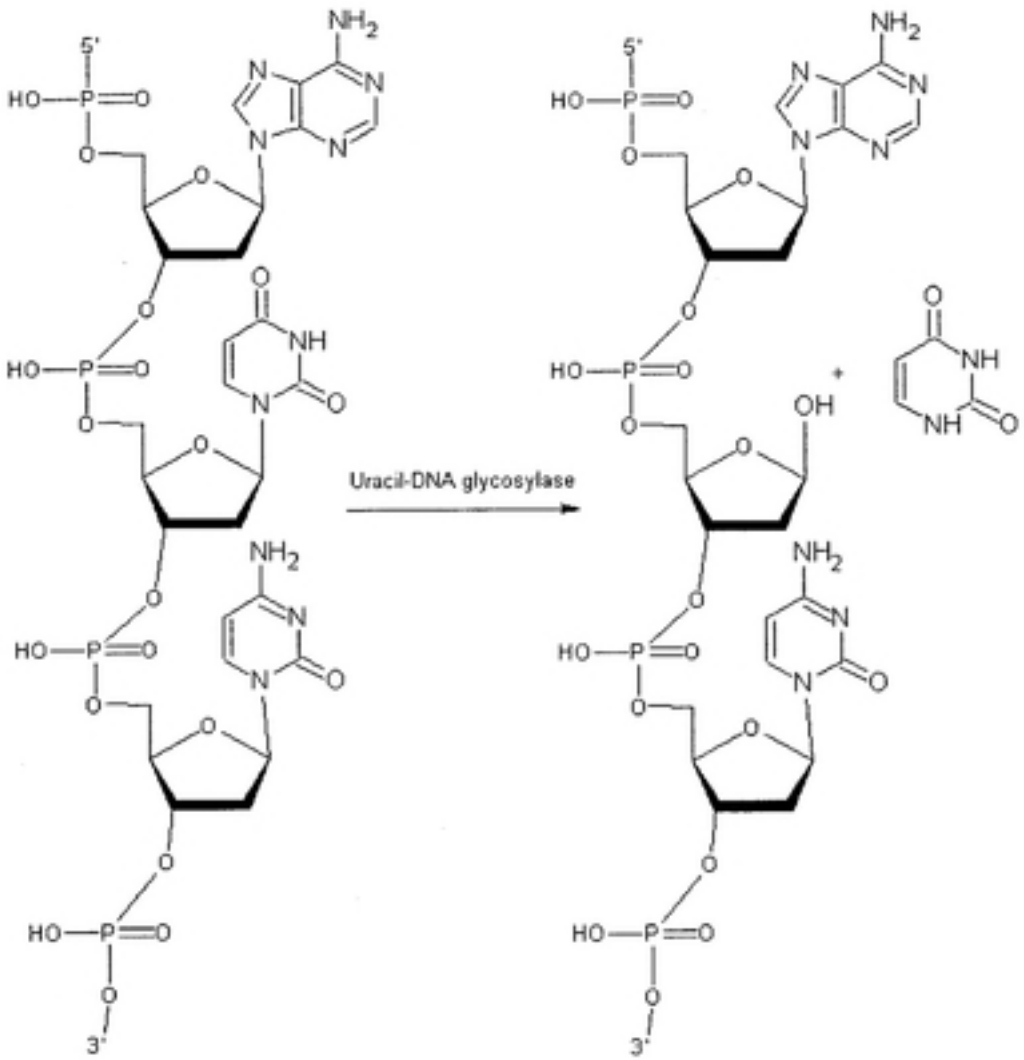


Figure 5. Illustrative example of the base excision repair of Uracil.

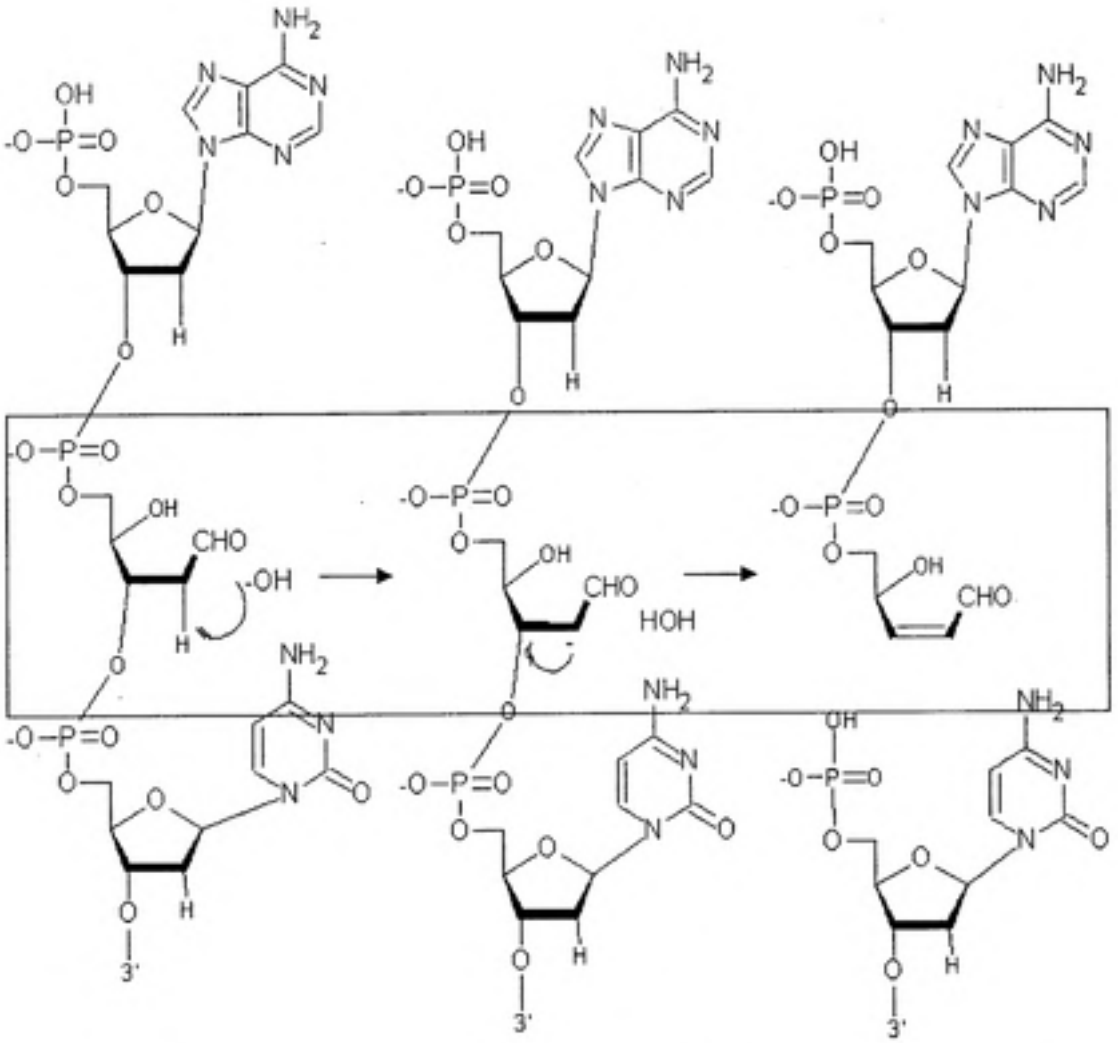


Figure 6. An AP site resulting in strand breakage of the DNA. The loss of a DNA base results in the AP site. β-elimination results in the formation of a double bond in the sugar and strand breakage occurs on the 3' side of the apurinic site.

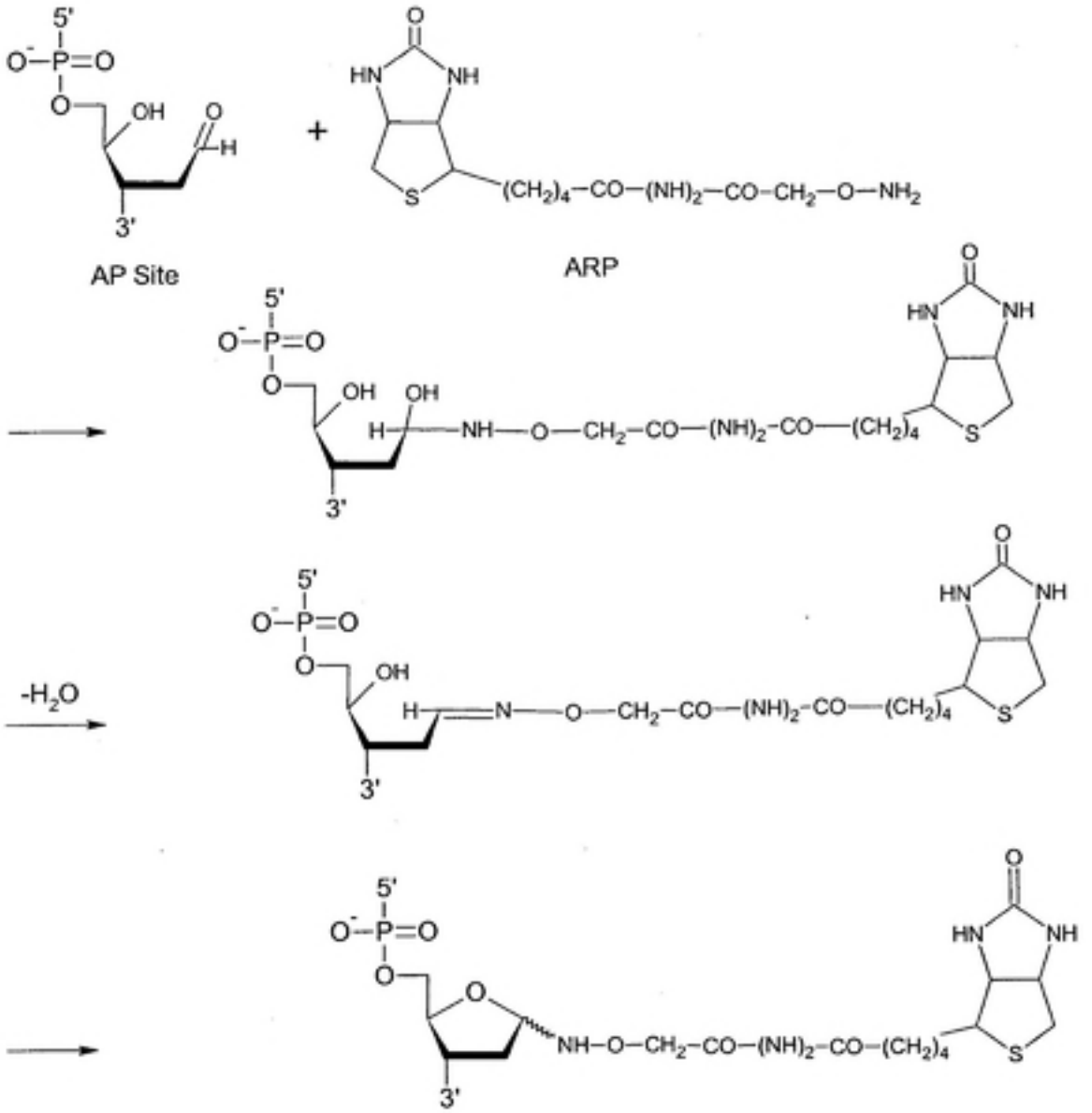


Figure 7. Reaction scheme of ARP with an AP sites.

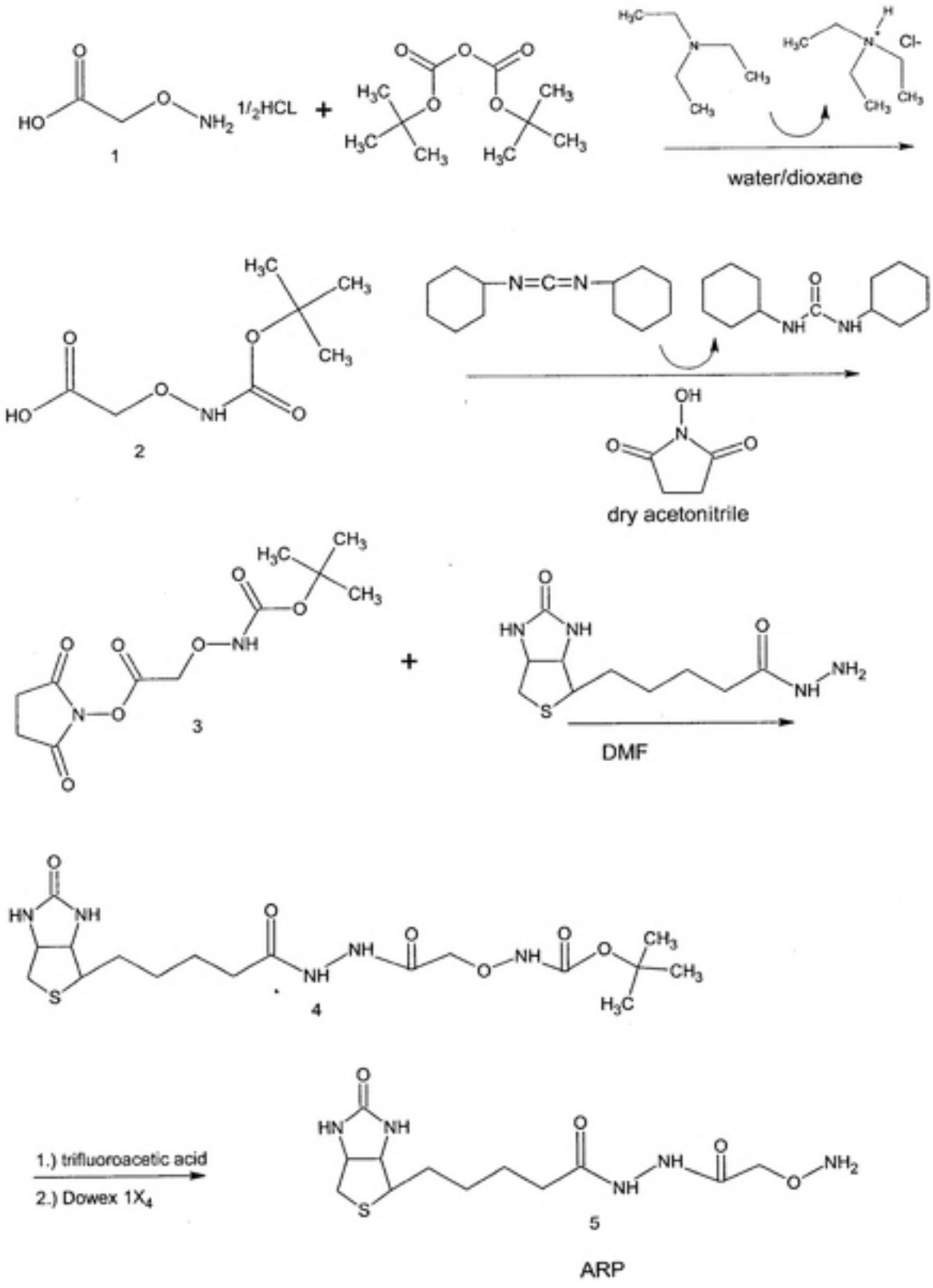


Figure 8. The synthesis of ARP

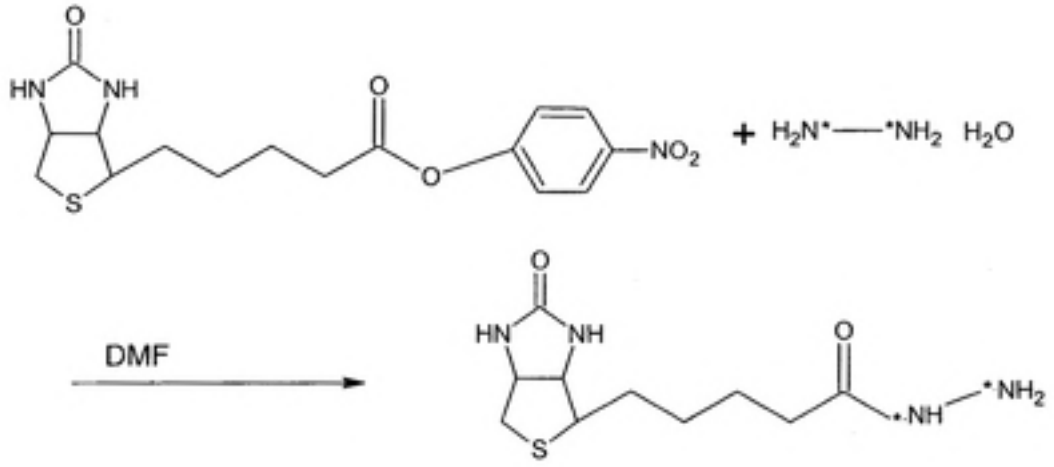


Figure 9. Synthesis of biotin hydrazide.

$$(CH_3)_2C{=}NONa + Br{*}CH_2{*}CO_2Na \rightarrow (CH_3)_2C{=}NO{*}CH_2{*}CO_2Na + NaBr$$

$$(CH_3)_2C{=}NO{*}CH_2{*}CO_2Na + HCl \rightarrow (CH_3)_2C{=}NO{*}CH_2{*}COOH + NaCl$$

$$(CH_3)_2C{=}NO{*}CH_2{*}COOH + H_2O + \tfrac{1}{2}\,HCl \rightarrow HOO{*}C{*}CH_2ONH_2\ 1/2HCl + (CH_3)_2CO$$

Figure 10. Synthesis of O-(carboxymethyl)hydroxylamine hemihydrochloride.

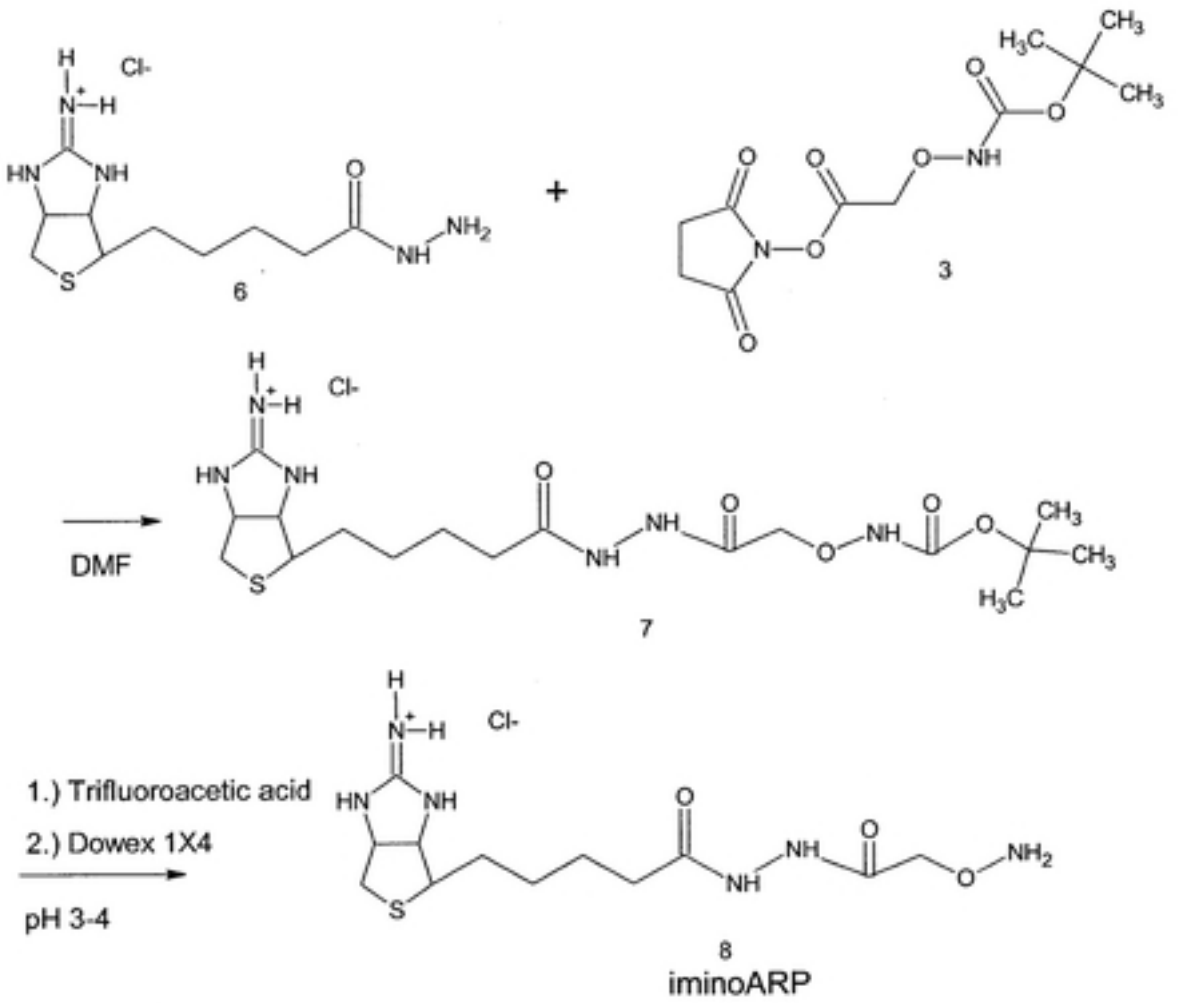


Figure 11. Synthesis of iminoARP.

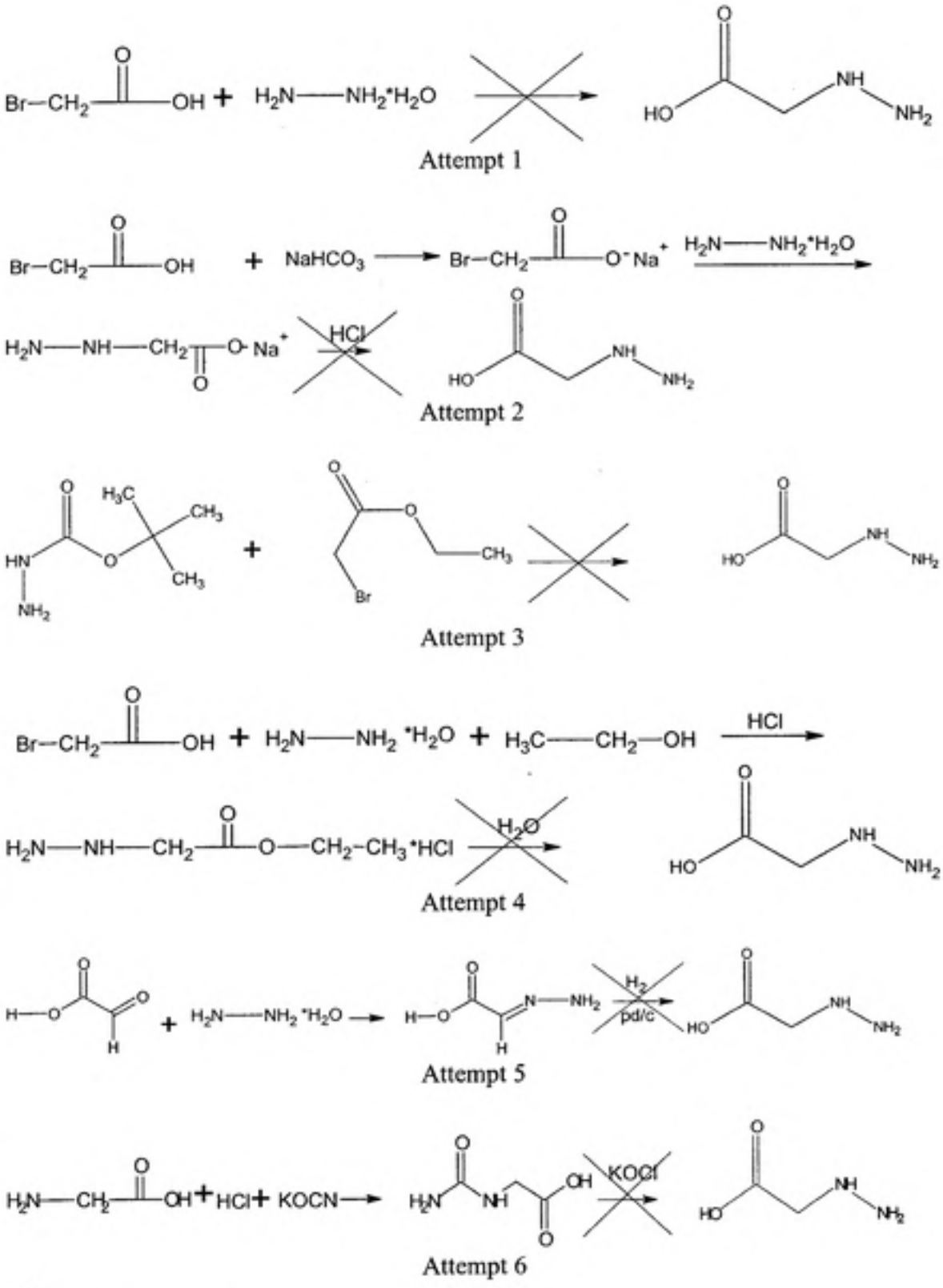


Figure 12. Attempted hydrazinoacetic acid syntheses.

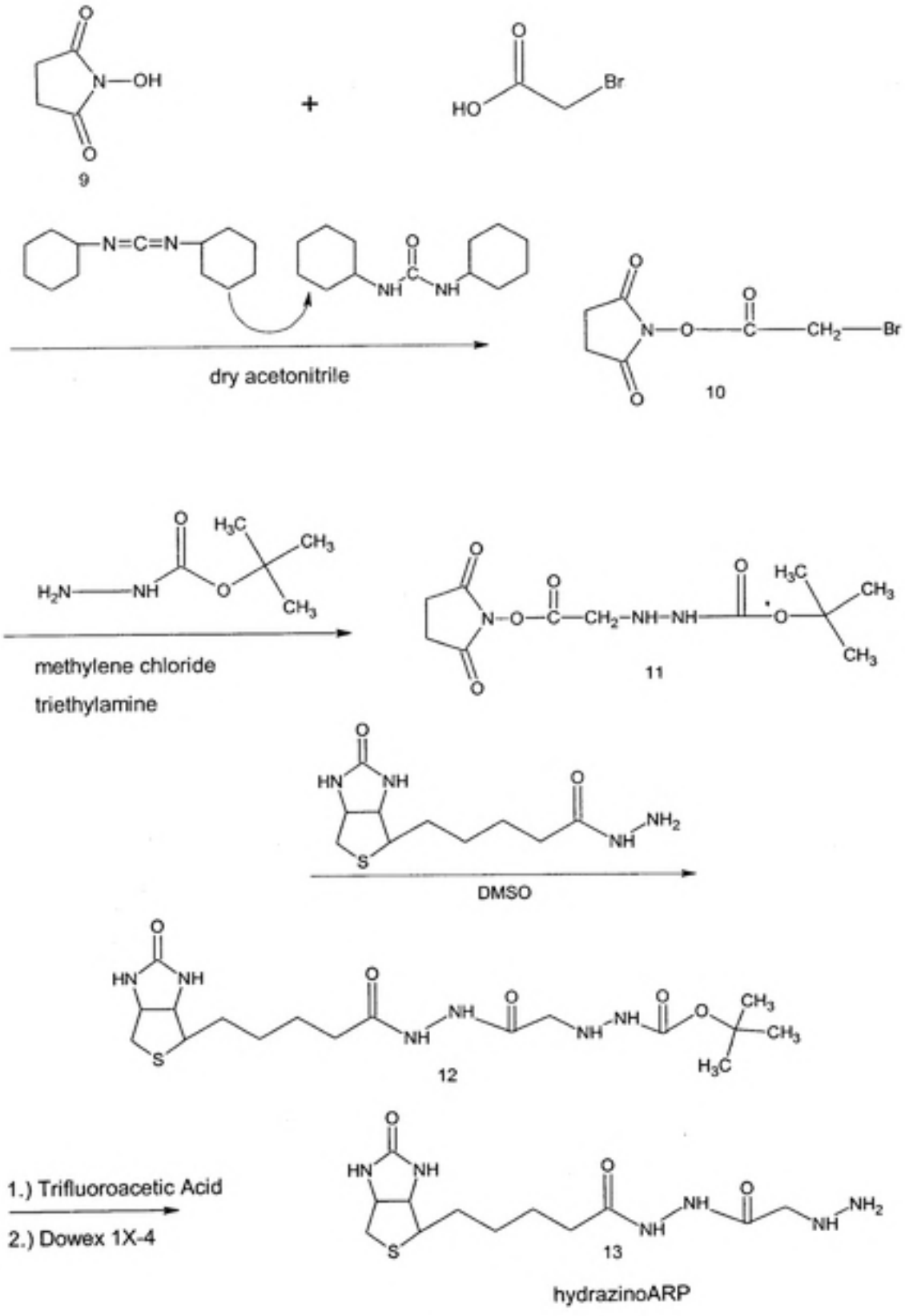


Figure 13. Synthesis of hydrazinoARP.

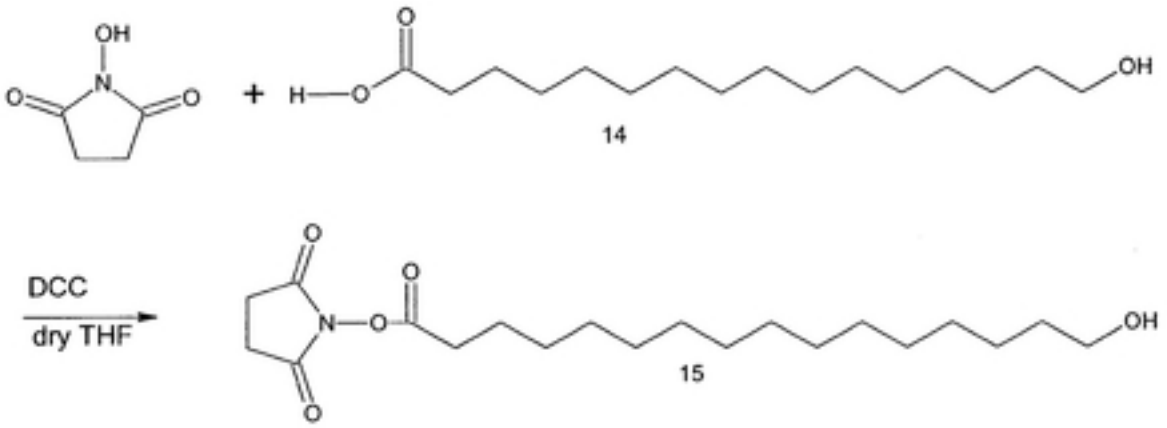


Figure 14. First reaction in the synthesis of the 16-hydroxyhexadecanoicARP.

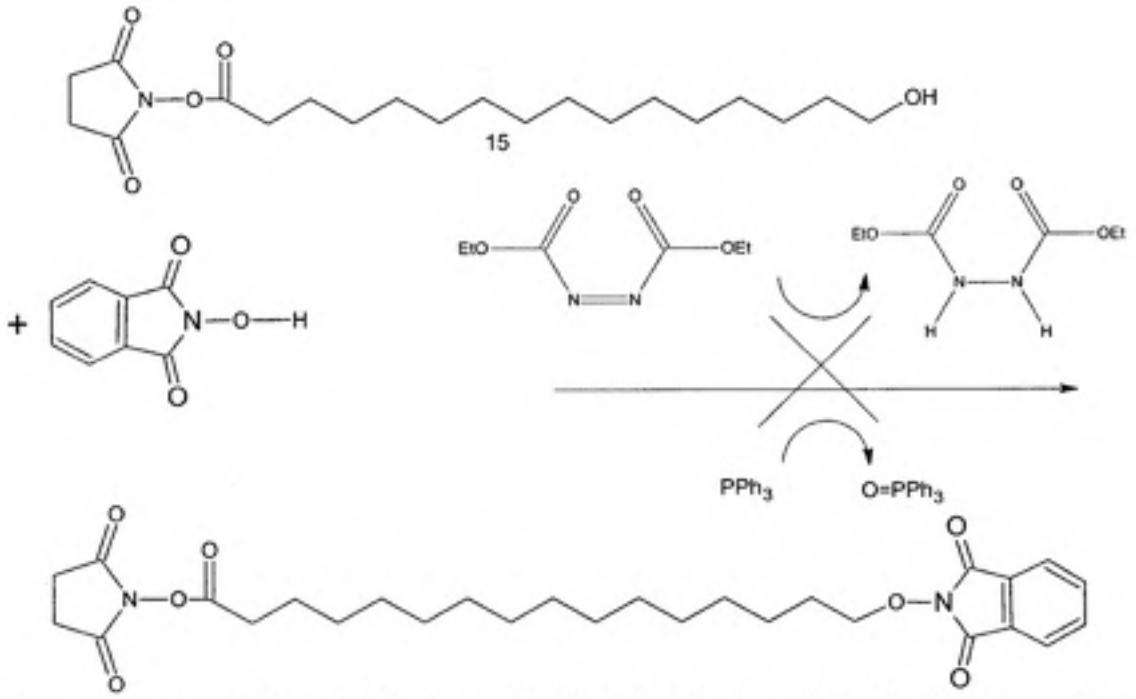


Figure 15. The addition of N-hydroxyphthalimide did not result in the formation of an ester with the alcohol group of 16-hydroxyhexadecanoic acid, but a transesterification reaction occurred instead.

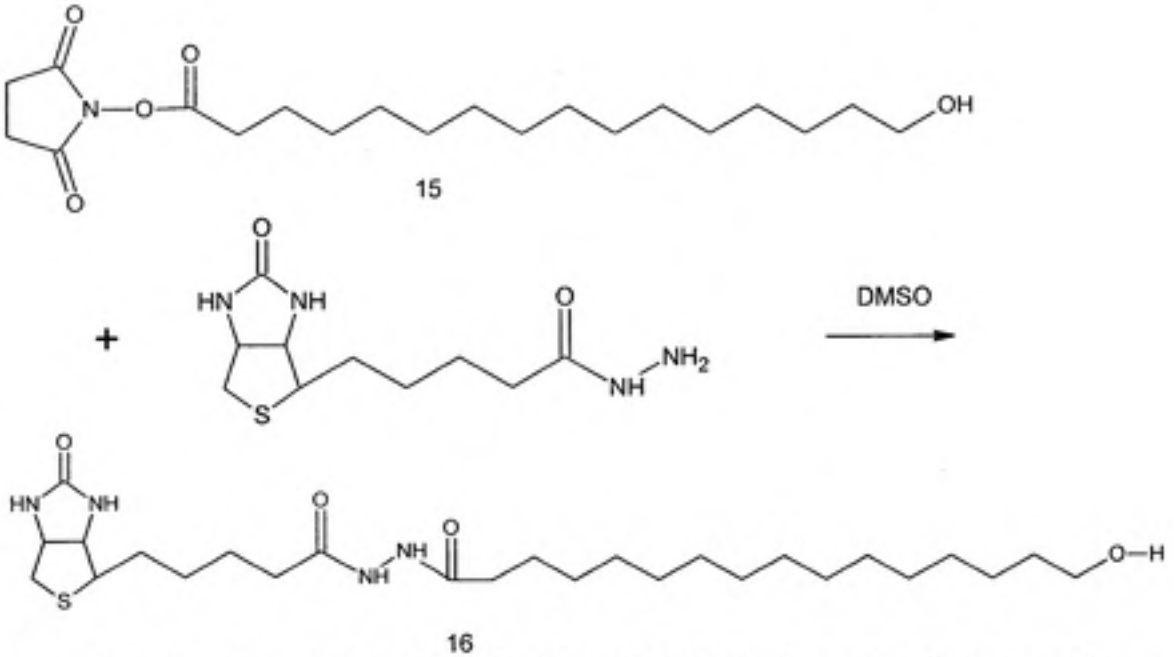


Figure 16. The addition of biotin hydrazide resulted in the formation of a less reactive amide bond which allowed for the formation of an ether bond with N-hydroxyphthalimide in the next synthesis step.

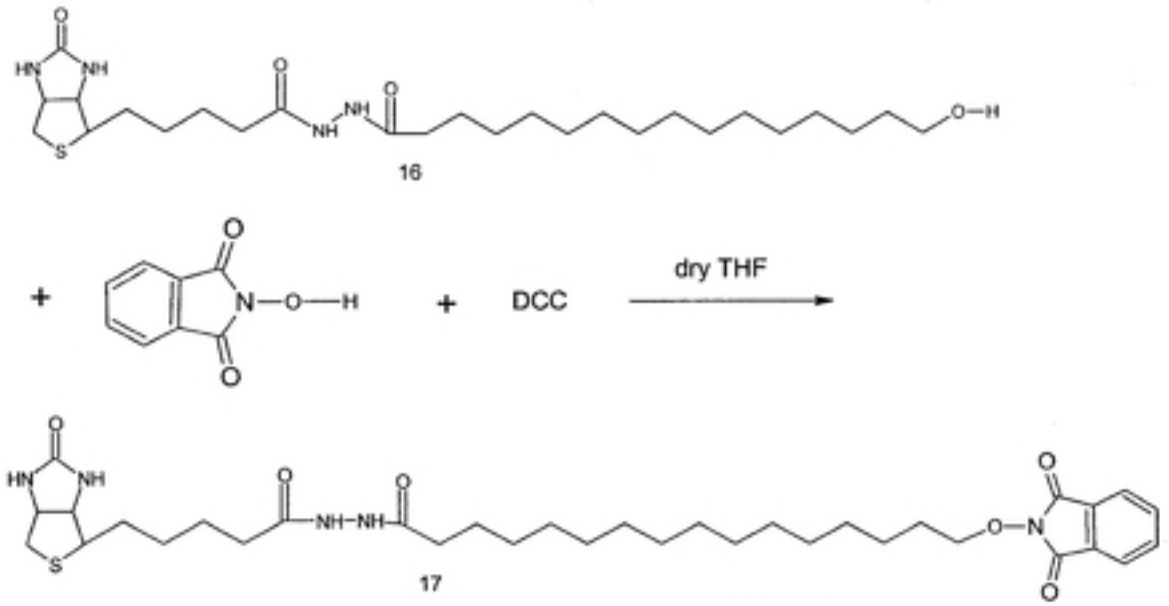
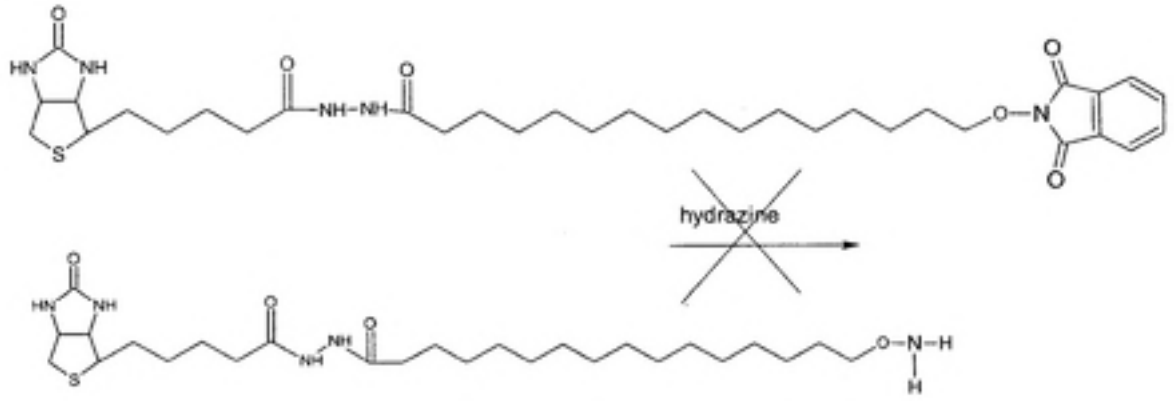


Figure 17. The second protection of 16-hydroxyhexadecanoic acid using N-hydroxyphthalimide was accomplished once the ester bond was converted to an amide bond.



16-hydroxyhexadecanoicARP

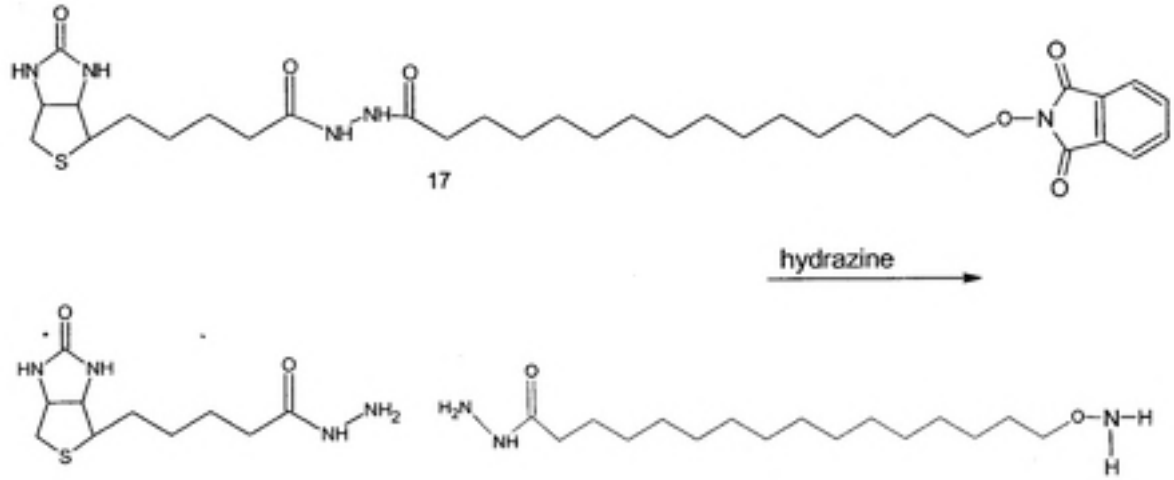
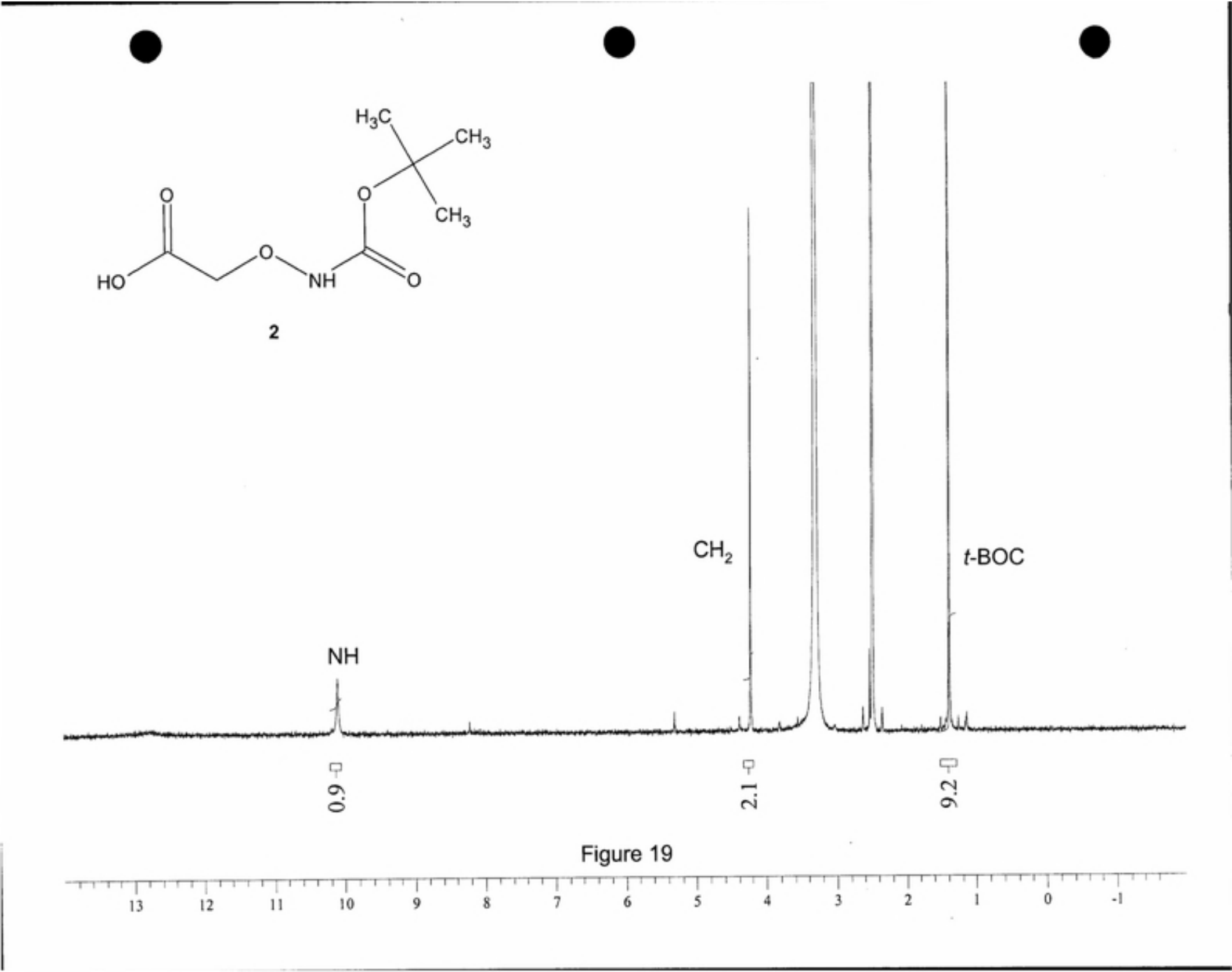


Figure 18. The addition of hydrazine in the final stage of the synthesis of the 16-hydroxyhexadecanoicARP did not result in the target ARP, but removed the biotin hydrazide from the compound.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CH3
CH3
O
O
HO
O
NH
O
2
CH2
t-BOC
NH
0.9
2.1
9.2
13
12
11
10
9
8
7
6
5
4
3
2
1
0
-1

Figure 19

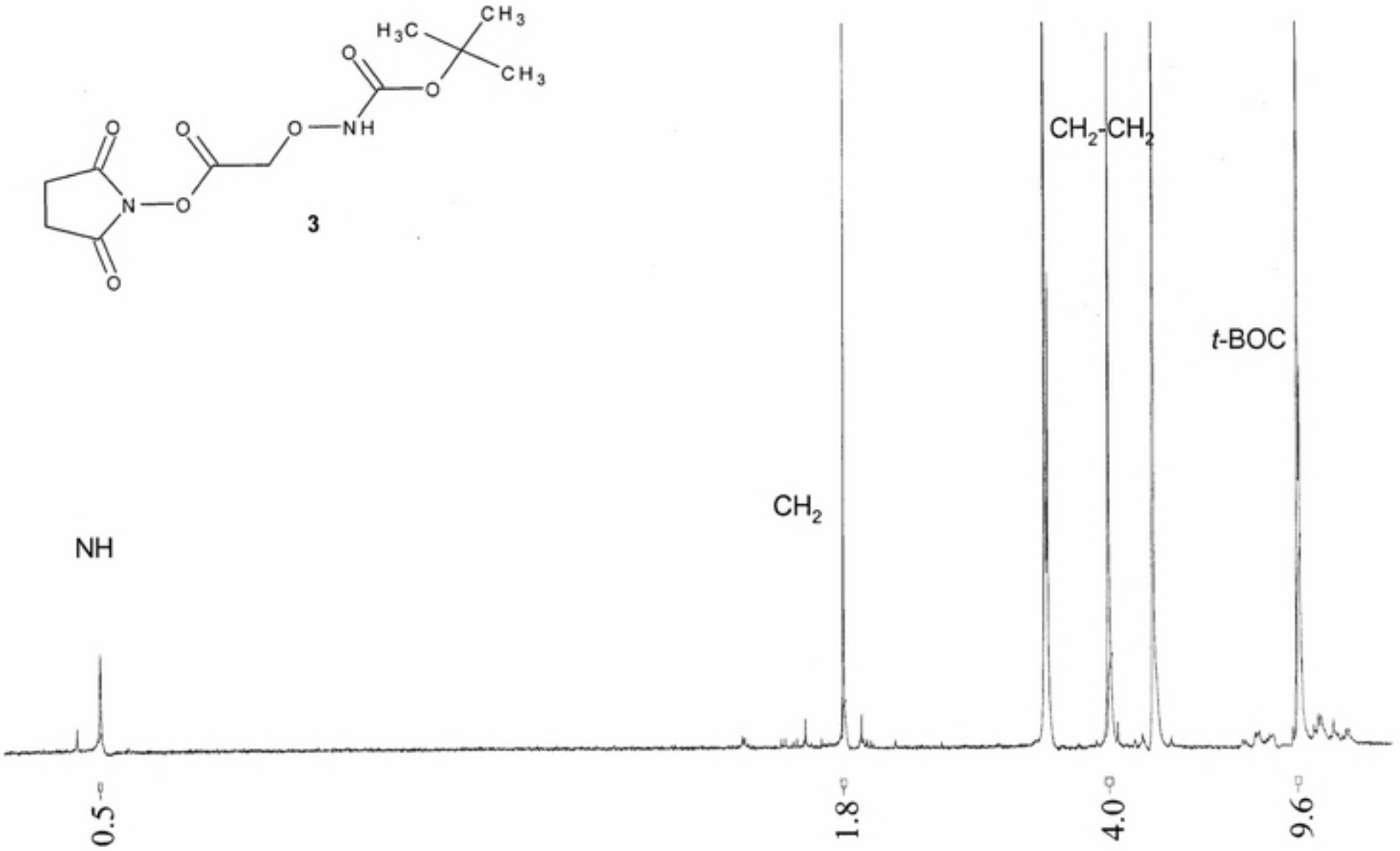
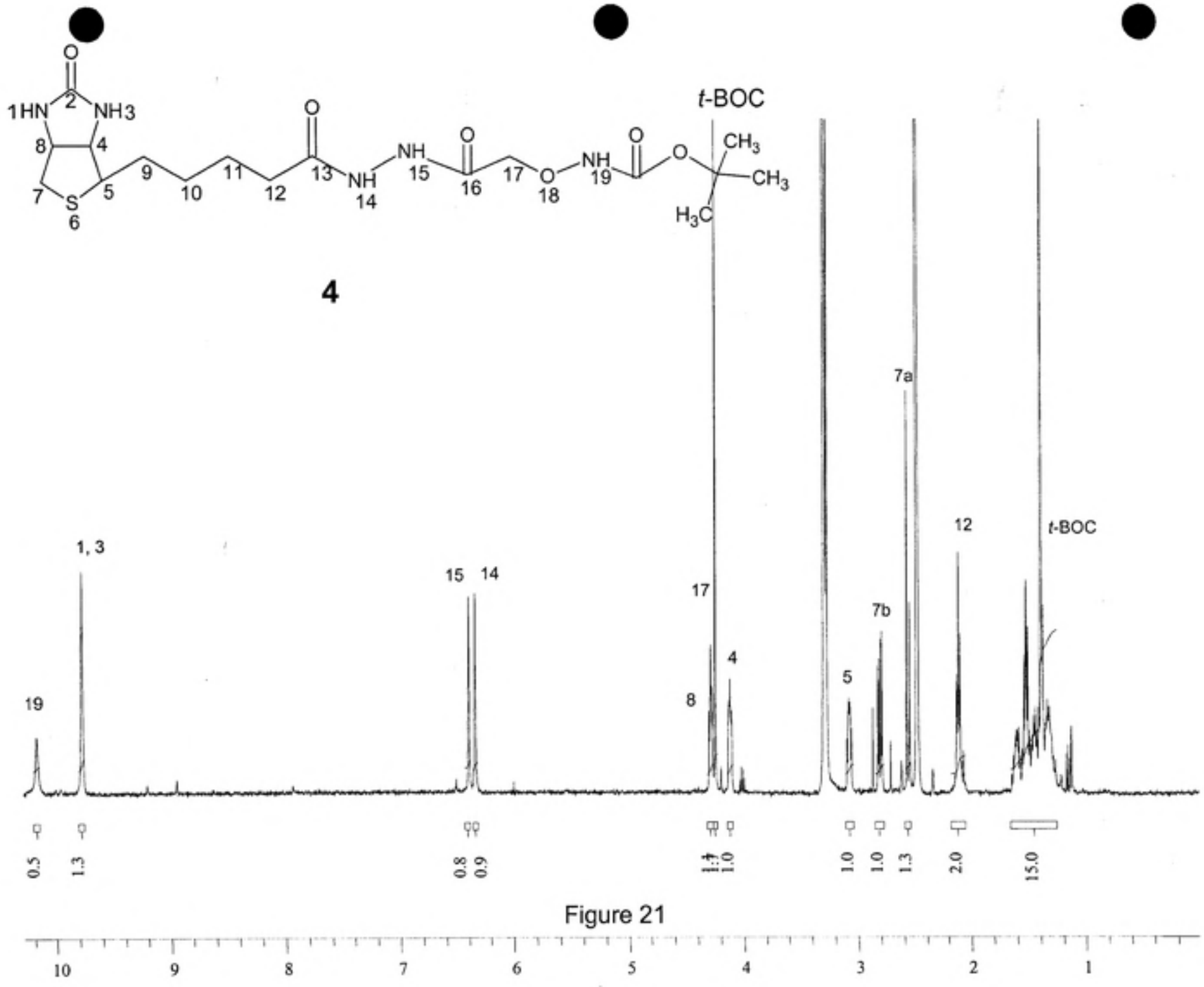


Figure 20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4
1, 3
15 14
17
8
4
19
5
7b
7a
12
t-BOC
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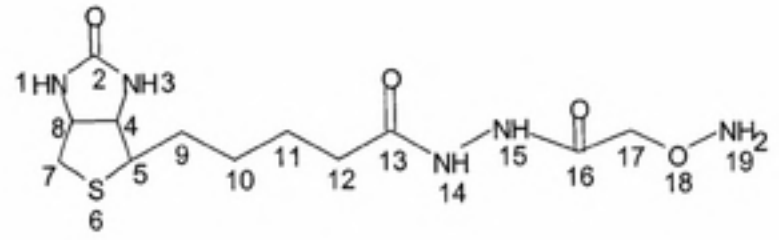
1.3
0.8
0.9
1.0
1.0
1.0
1.3
2.0
15.0
10
9
8
7
6
5
4
3
2
1

Figure 21



5

ARP

Figure 22

ARP

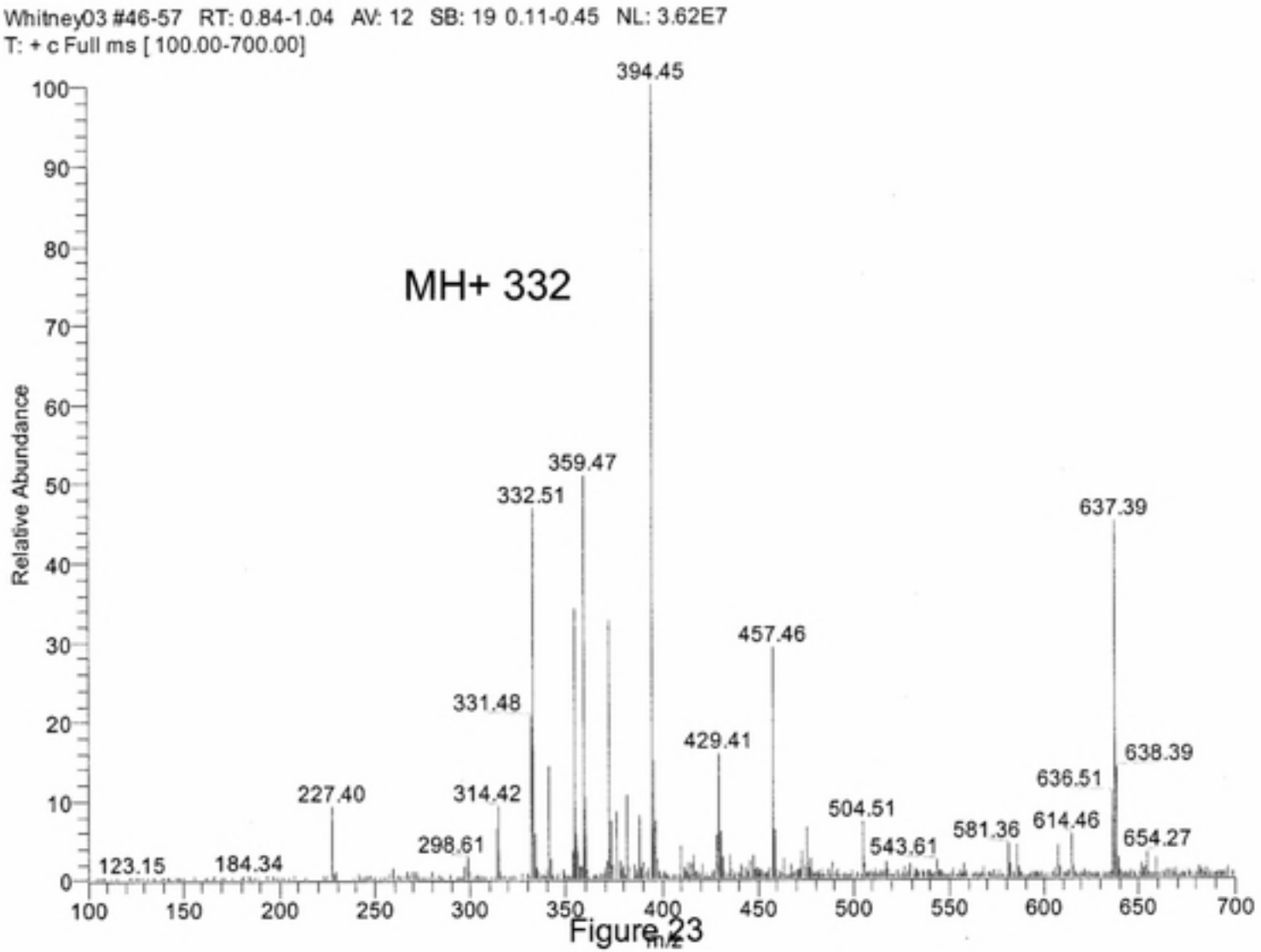
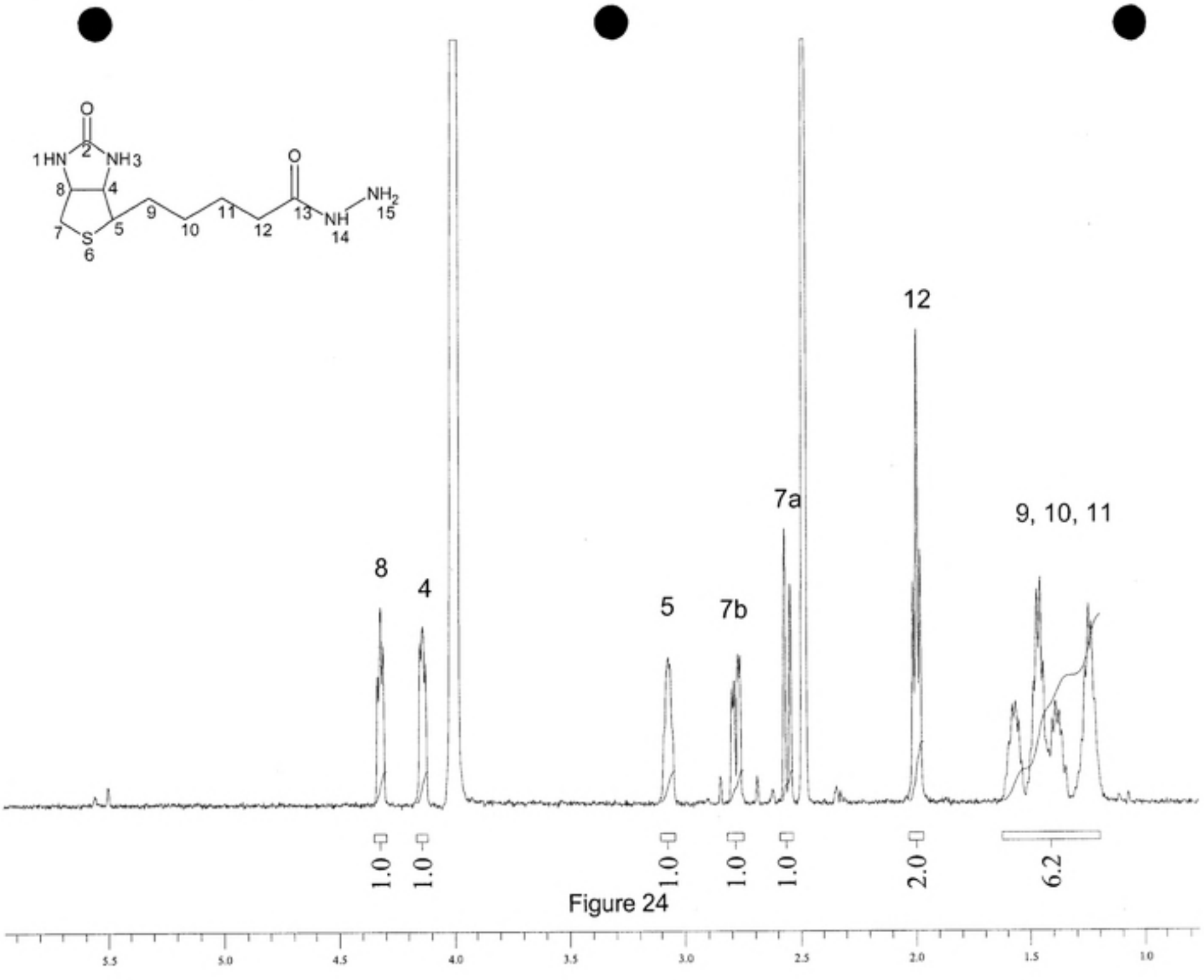


Figure 23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2
NH3
8
4
9
10
11
12
13
NH
14
NH2
15
7
S
6
5
8
4
5
7b
7a
12
9, 10, 11
1.0
1.0
1.0
1.0
1.0
2.0
6.2
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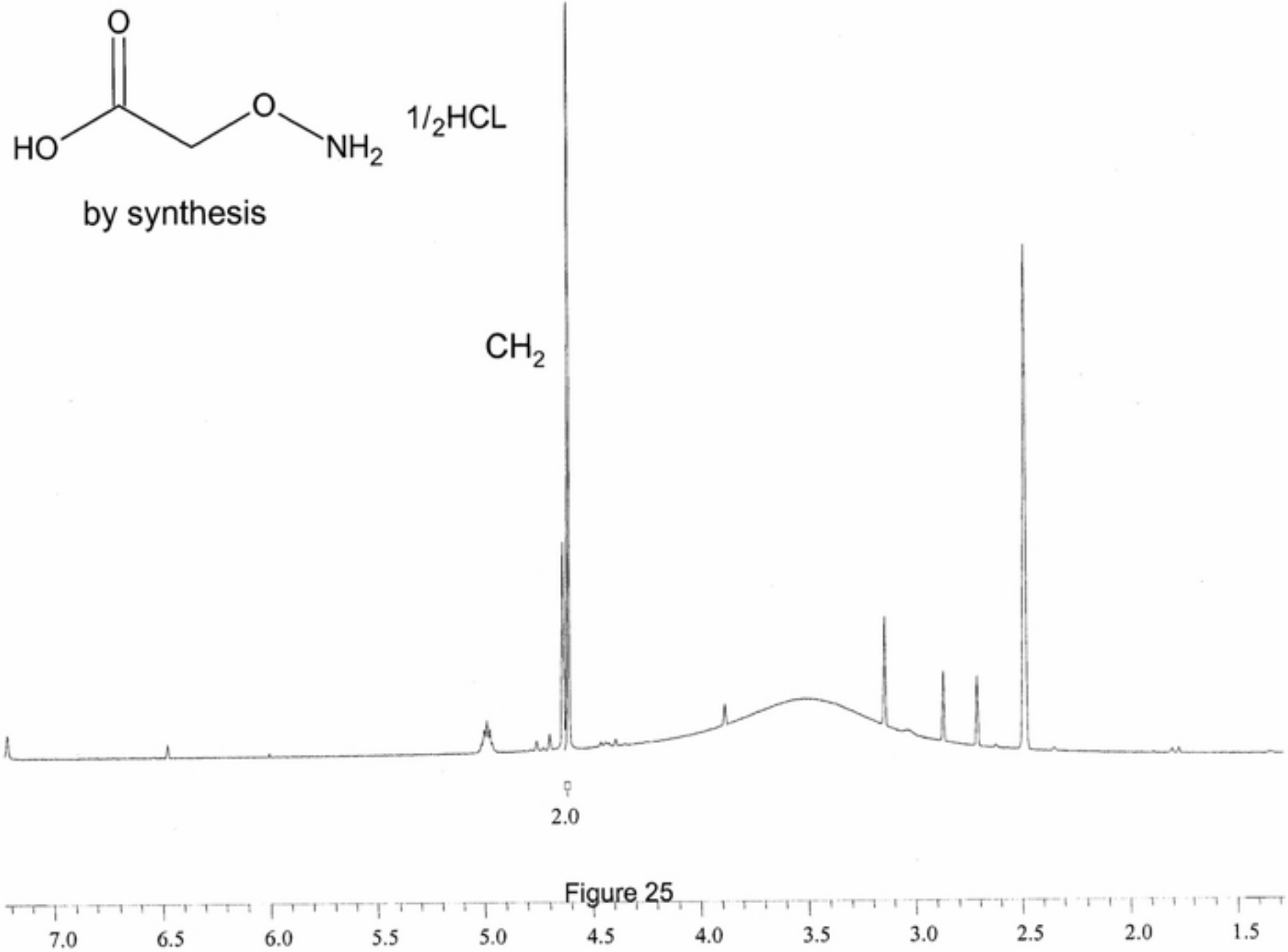
3.5
3.0
2.5
2.0
1.5
1.0

Figure 24


O
HO
O
NH2
1/2HCL
by synthesis
CH2
2.0
7.0
6.5
6.0
5.5
5.0
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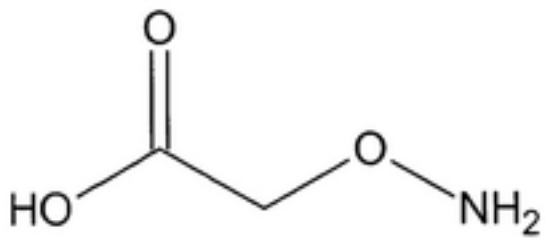
3.5
3.0
2.5
2.0
1.5

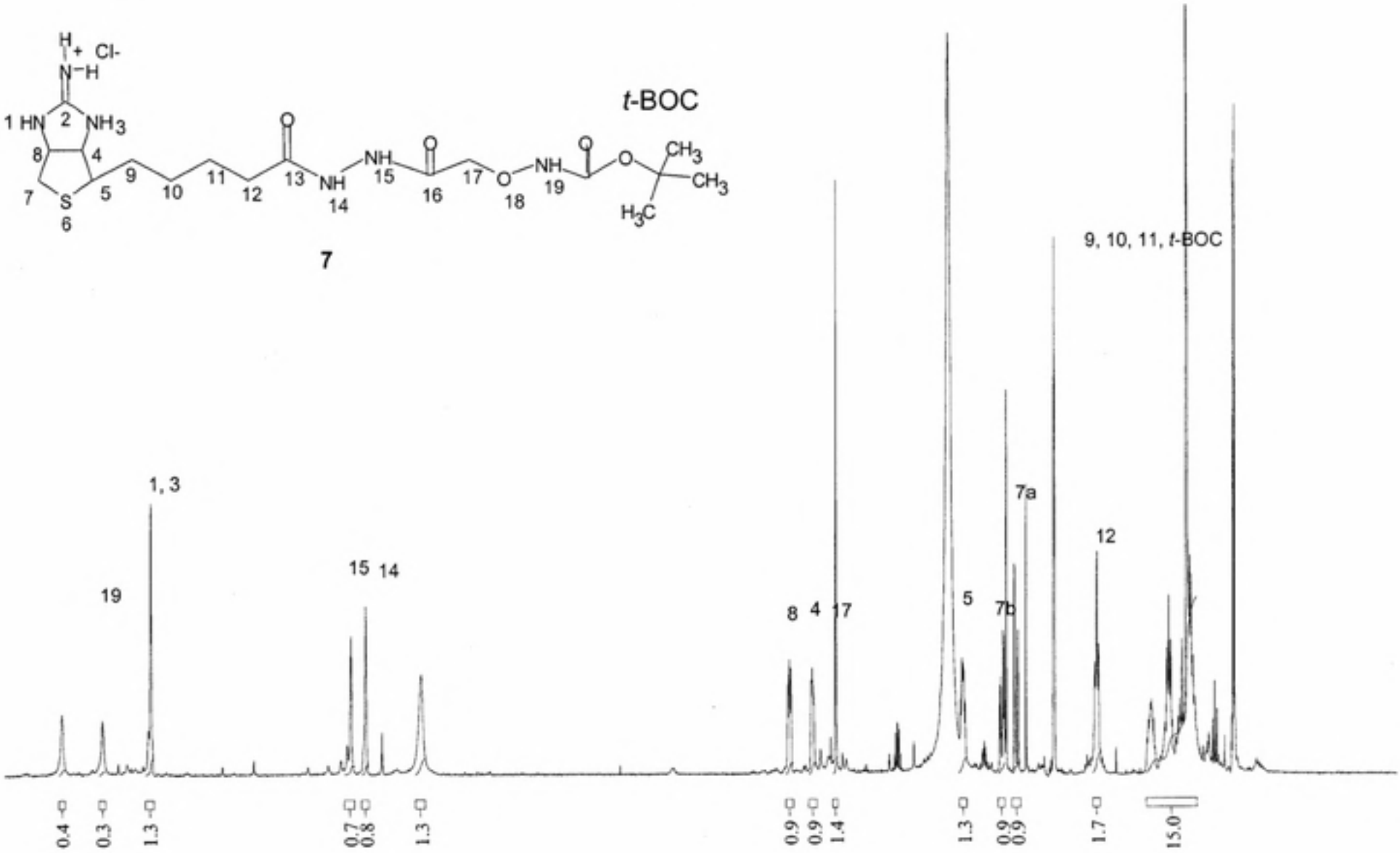
Figure 25



1/2HCL

standard

Figure 26


t-BOC
7
1, 3
19
15 14
8 4 17
5
7b
7a
12
9, 10, 11, t-BOC
0.4
0.3
1.3
0.7
0.8
1.3
0.9
0.9
1.4
1.3
0.9
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1.7
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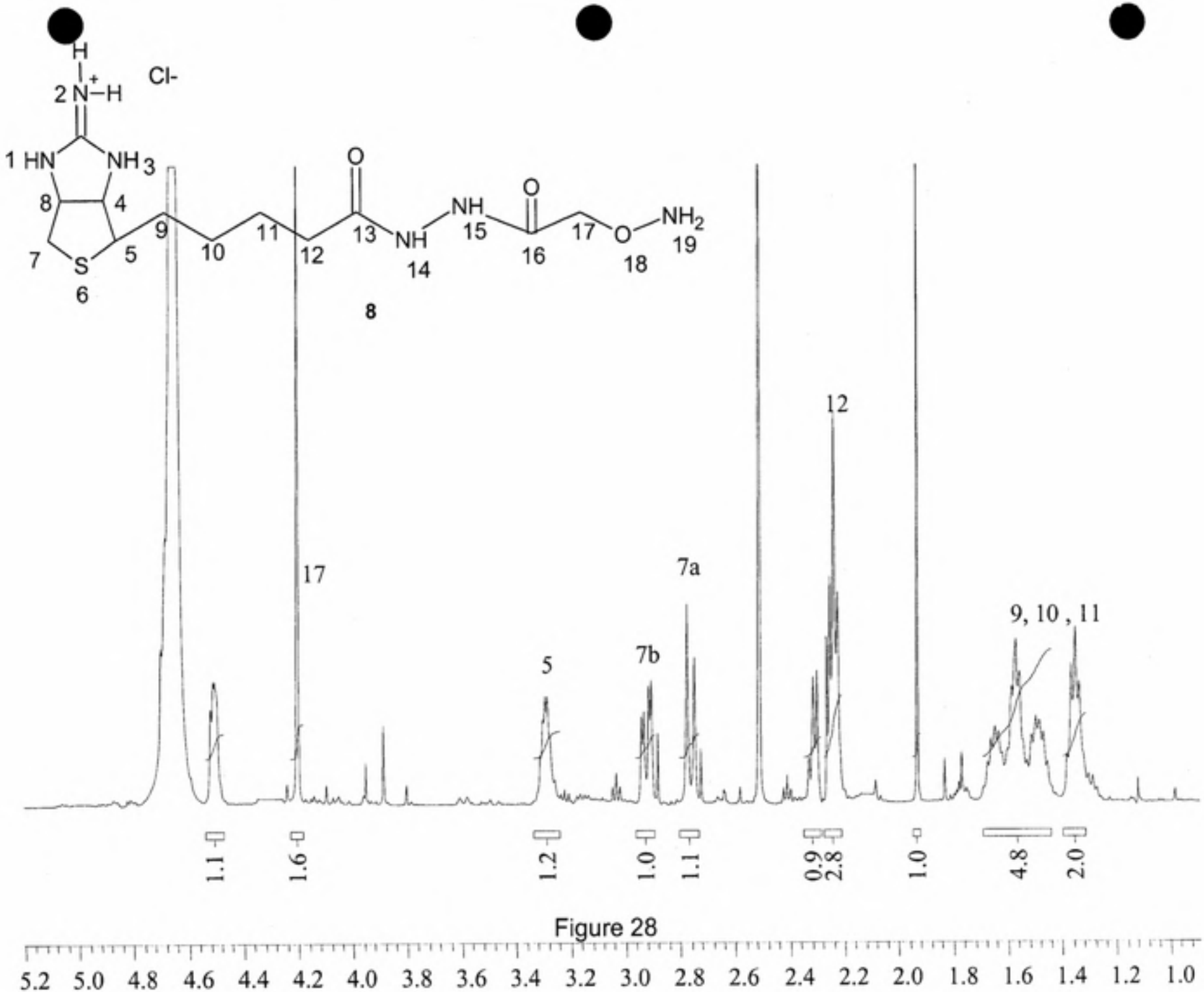
10
9
8
7
6
5
4
3
2
1
0

Figure 27


Cl-
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17
7a
7b
5
12
9, 10 , 11
1.1
1.6
1.2
1.0
1.1
0.9
2.8
1.0
4.8
2.0
5.2 5.0 4.8 4.6 4.4 4.2 4.0 3.8 3.6 3.4 3.2 3.0 2.8 2.6 2.4 2.2 2.0 1.8 1.6 1.4 1.2 1.0

Figure 28

IminoARP

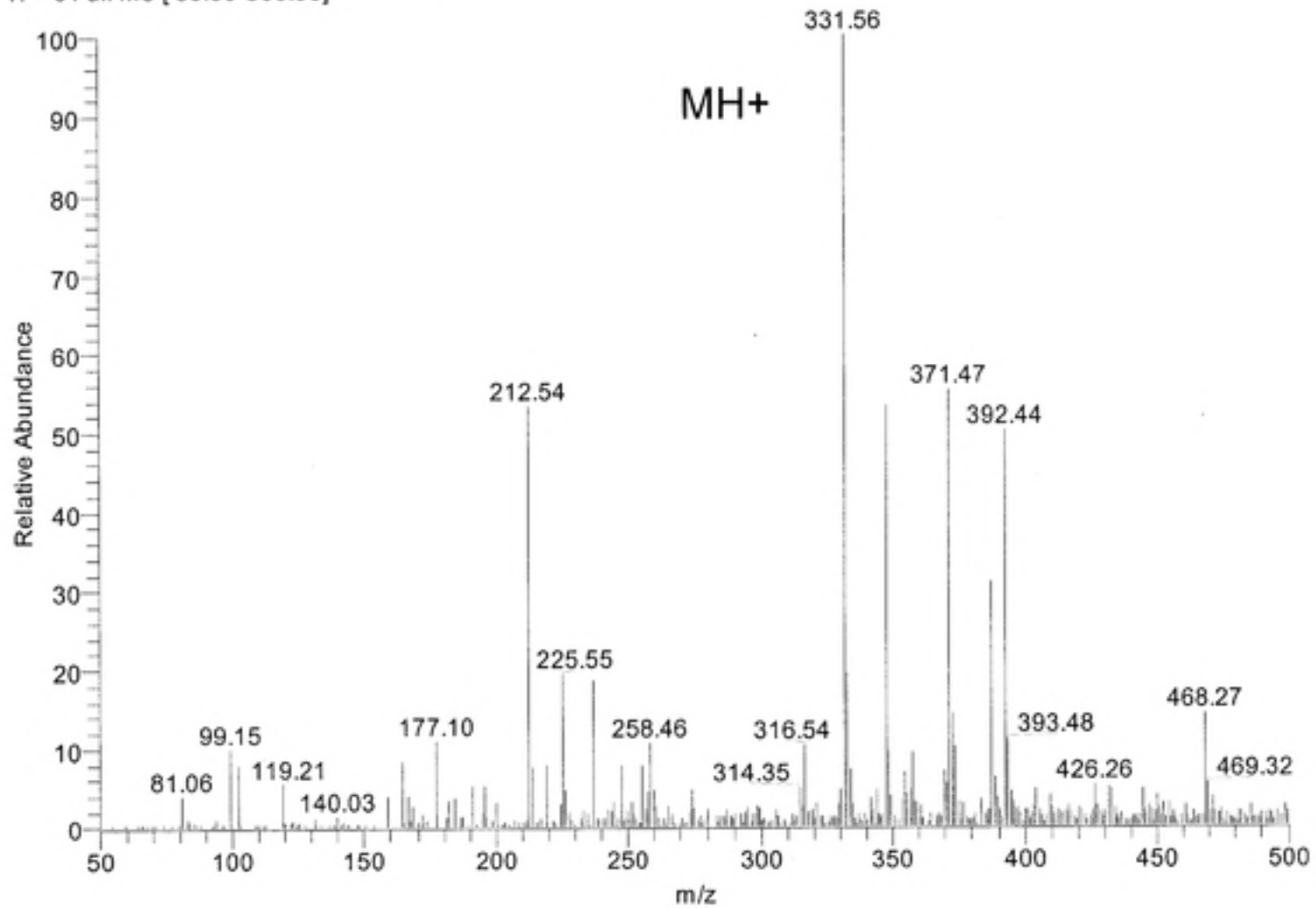
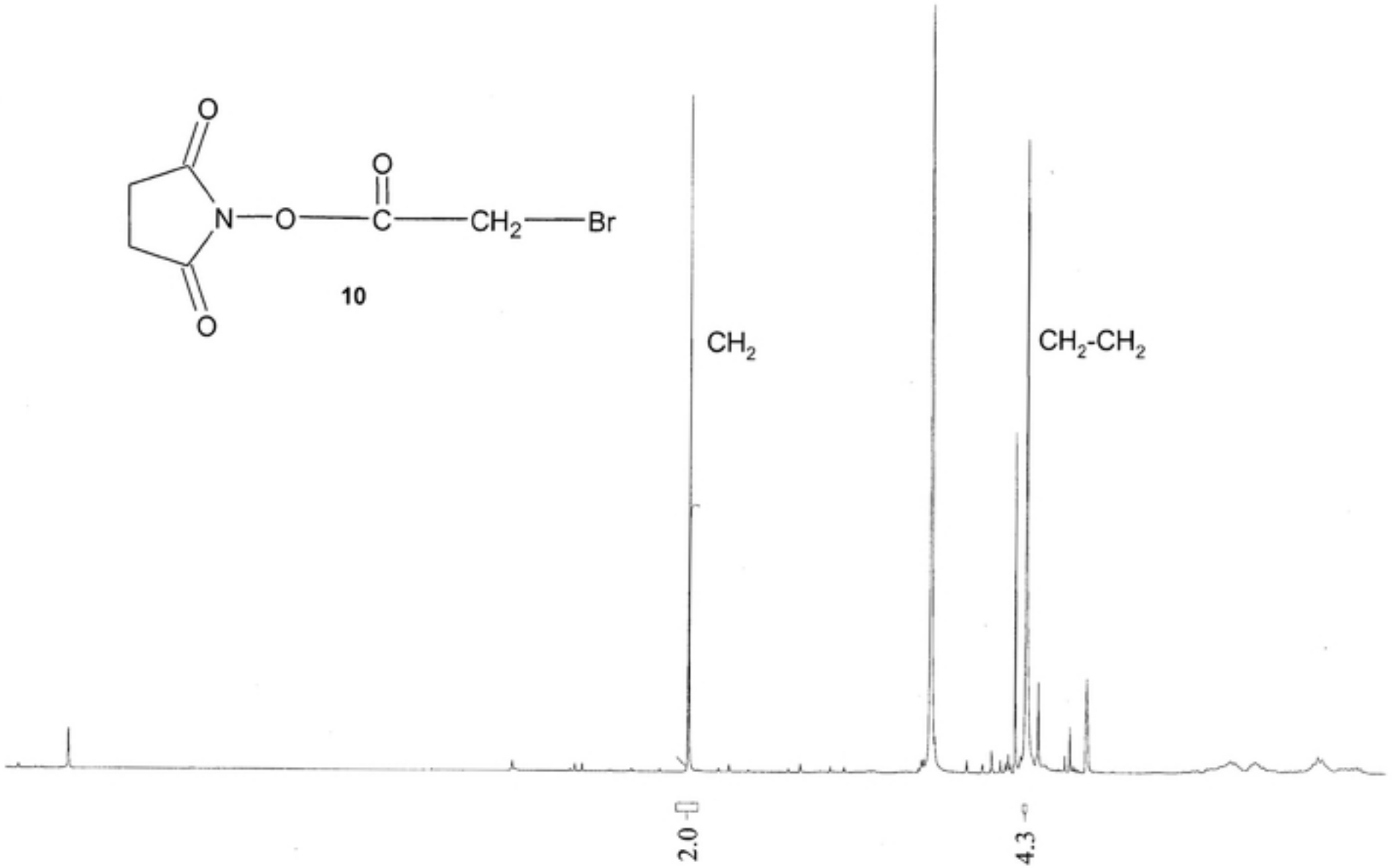


Figure 29


O
O
N
O
C
CH2
Br
O
10
CH2
CH2-CH2
2.0
4.3
8.0
7.5
7.0
6.5
6.0
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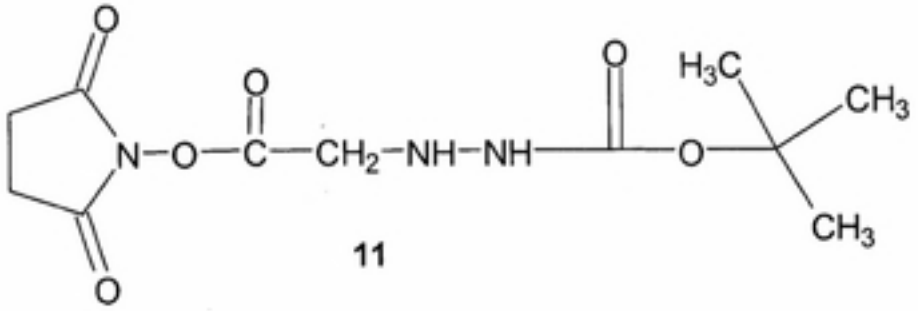
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0

Figure 30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O
N–O–C–CH2–NH–NH
O
O
H3C
CH3
CH3
O

11

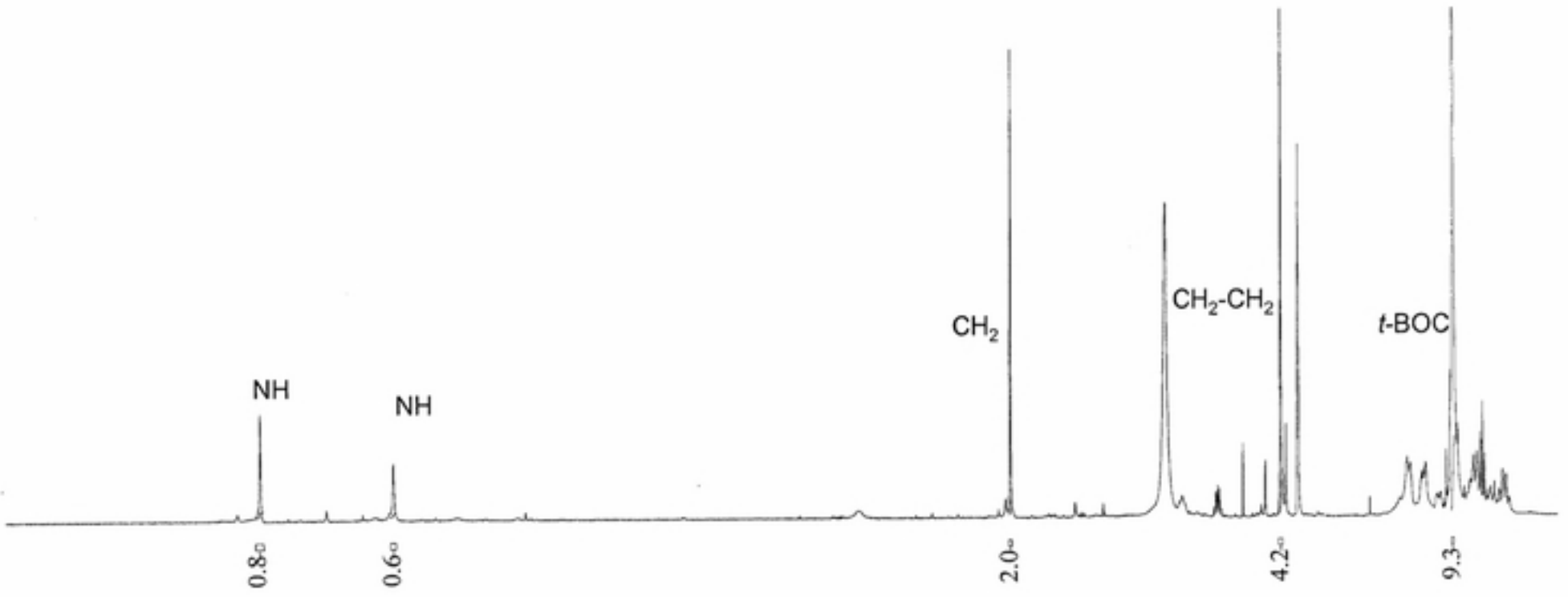

NH
NH
CH2
CH2-CH2
t-BOC
0.8
0.6
2.0
4.2
9.3

Figure 31

Date : 04-Apr-102 16:35
:cinimideester, C11H17N3O6, MW=287, NBA/MeCl2
3-2000, 20s, cycle=14, 200-600 amu
Ion Mode : FAB+
-mal Ion [MF-Linear]
Scan# : (2,4) Temp : 51.0 deg.C
Int. : 89.60
200.0000 to 600.0000 Cut Level : 0.00 %

N-hydroxysuccinimide ester of N^1-carboxymethyl-tertiary butyl carbazate (FAB)

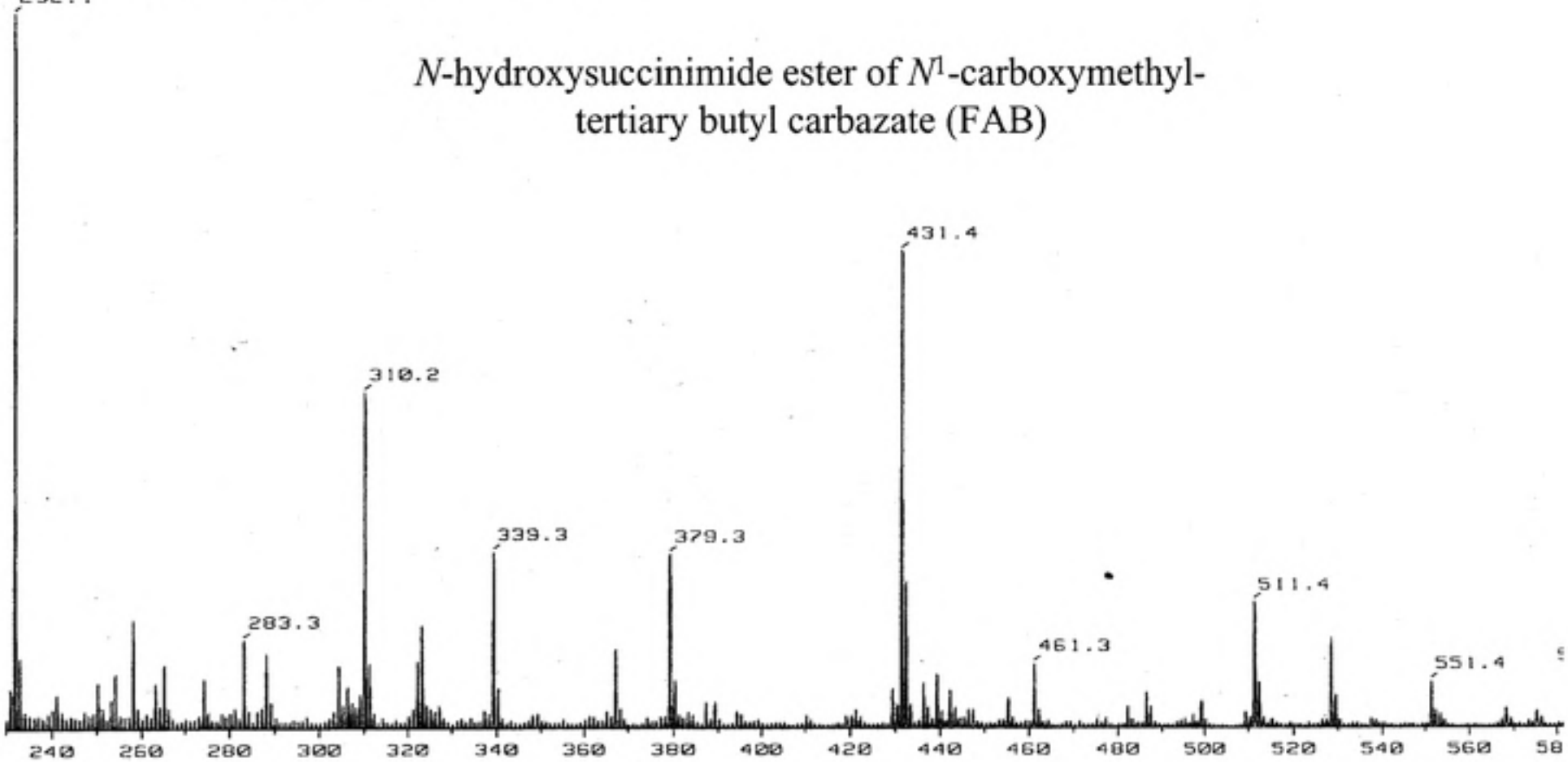


Figure 32

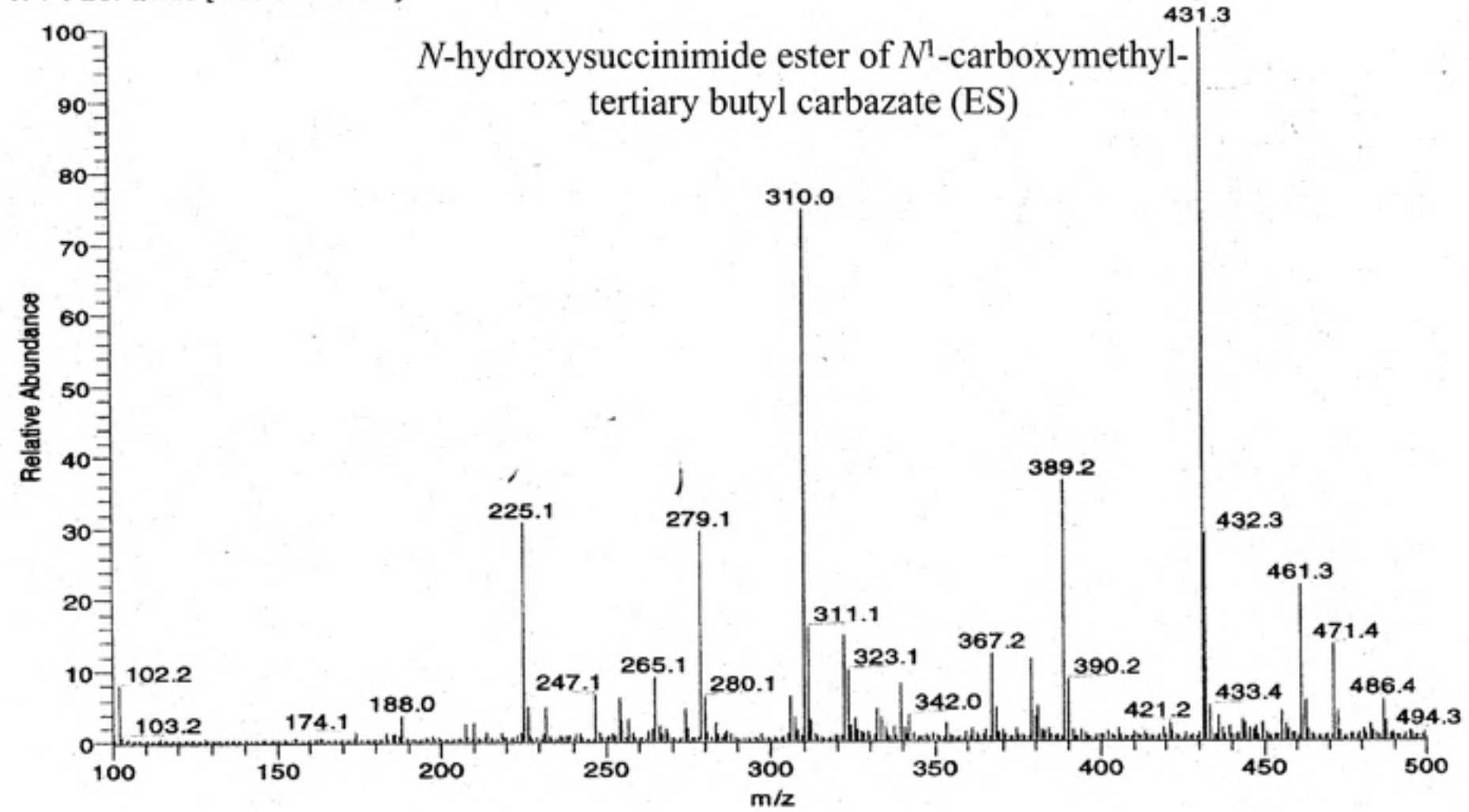

Whitney03 #55-66 RT: 0.91-1.09 AV: 12 NL: 1.27E7
T: + c ESI Q1MS [100.00-500.00]
N-hydroxysuccinimide ester of N1-carboxymethyl-tertiary butyl carbazate (ES)
Relative Abundance
m/z
431.3
310.0
389.2
225.1
279.1
432.3
461.3
311.1
471.4
323.1
367.2
390.2
265.1
247.1
280.1
342.0
433.4
486.4
421.2
494.3
102.2
103.2
174.1
188.0

Figure 33

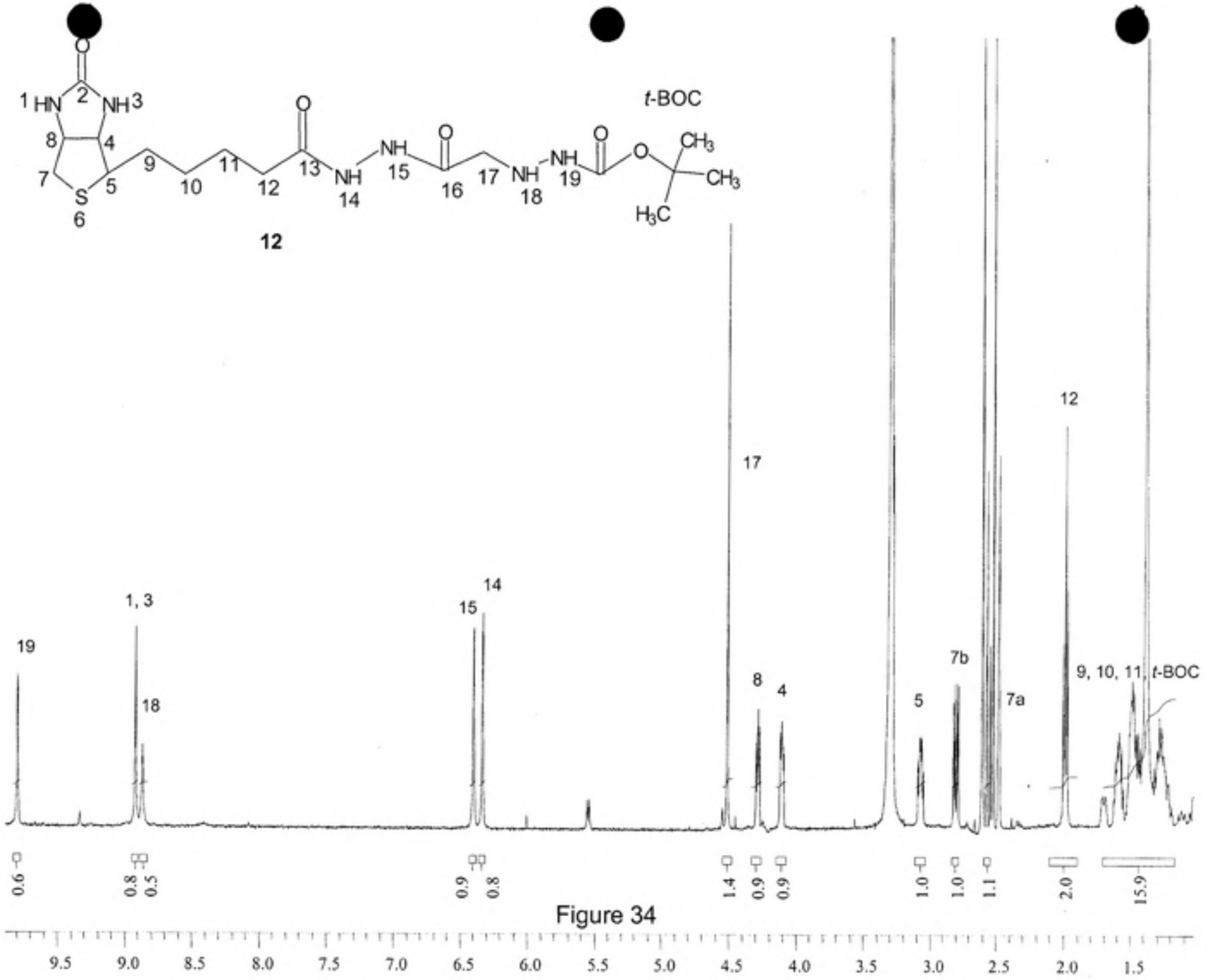
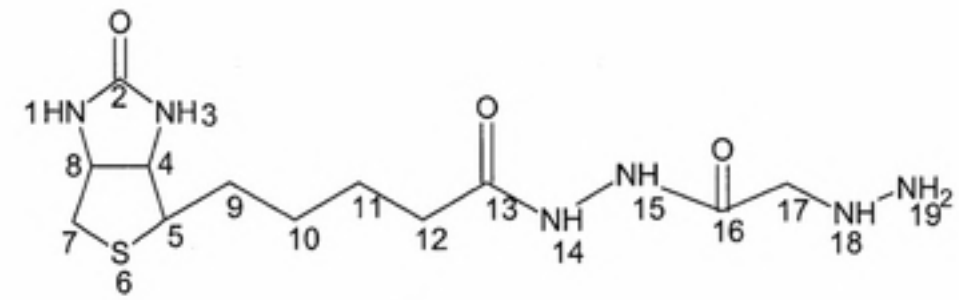

t-BOC
12
19
1, 3
18
15
14
17
8
4
5
7b
7a
12
9, 10, 11, t-BOC
0.6
0.8
0.5
0.9
0.8
1.4
0.9
0.9
1.0
1.0
1.1
2.0
15.9
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5

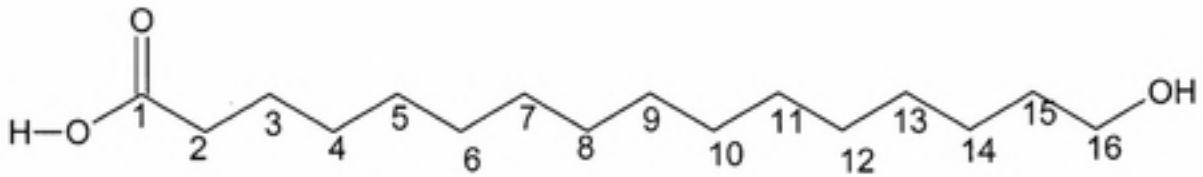
Figure 34


O
1HN
2
NH3
8
4
9
11
13
O
NH
15
O
NH2
19
17
NH
18
7
S
6
5
10
12
NH
14
16

13

4, 17
12
15
14
7a
7b
1, 3
8
9, 10, 11
5
0.9
0.9
1.0
1.1
2.7
1.0
1.1
1.1
2.0
6.4
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5

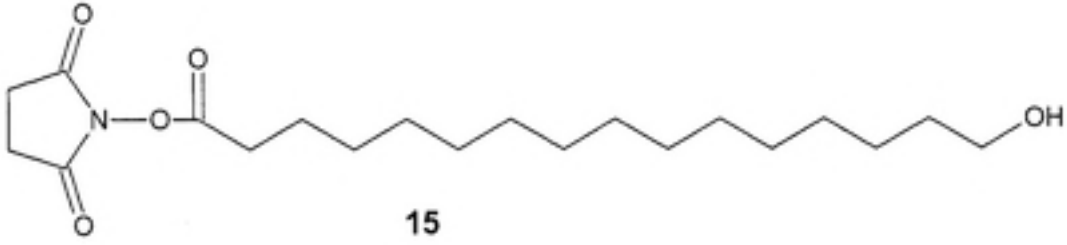
Figure 35


O
H—O
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
OH

14

(CH2)5-(CH2)13
CH2OH
CH2COOH
CH2CH2CH2OH
CH2CH2CH2COOH
1.7
2.0
4.2
3.9
18.6
4.4 4.2 4.0 3.8 3.6 3.4 3.2 3.0 2.8 2.6 2.4 2.2 2.0 1.8 1.6 1.4 1.2 1.0 0.8 0.6 0.4

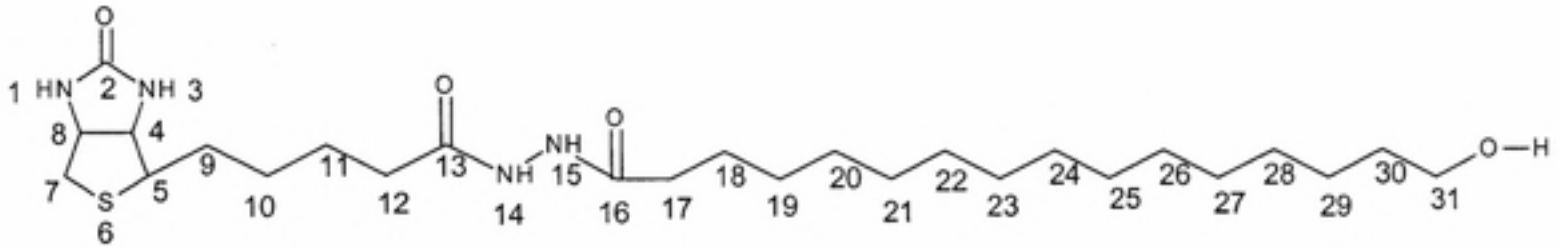
Figure 36


O
O
N
O
O
OH

15

(CH2)5-(CH2)13
CH2OH
CH2-CH2
CH2COOH
CH2CH2CH2OH
CH2CH2CH2COOH
2.0
3.8
2.1
4.3
4.6
18.5
4.2
4.0
3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0

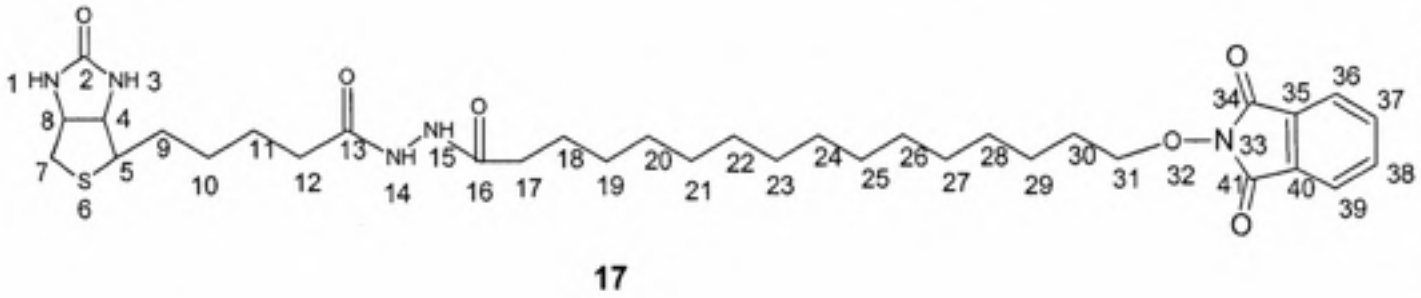
Figure 37


O
1 HN 2 NH 3
8 4
7 S 5
6
9 10 11 12 13 O NH 14 NH 15 O 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 O—H

16

15
14
8
4
5
7b
31
17
7a
12
9-11, 18-30
2.1
1.5
1.3
1.0
1.1
2.9
1.0
3.0
1.6
32.1
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5

Figure 38



17

36, 37, 38, 39
1, 3
14, 15
8
4
31 masked
by HOD
5
7b
7a
12, 17
9-11, 18-30
1.5
4.0
1.7
0.5
1.5
0.8
2.7
1.3
0.9
4.1
34.1
10.5
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0

Figure 39

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